CIGARETTE SMOKING AND IMMUNITY:

STUDY OF AN EXPERIMENTAL MURINE MODEL

by

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SUMMARY

In order to investigate the possibility that damage to the immune system can be produced by inhalation of cigarette smoke, and that this can conceivably expedite respiratory infection and neoplasia, the immune responses of mice exposed to cigarette smoke were studied. While the method of exposure to cigarette smoke produced peak carboxyhaemoglobin levels of approximately 20%, which rapidly declined, this was considerably less than acute toxic doses. Mice chronically exposed to cigarette smoke had body-weights and food consumptions less than control mice. Haemoglobin levels were initially elevated by exposure to cigarette smoke but after prolonged exposure the levels fell to 5% below control values. The most marked haematological change produced by exposure to cigarette smoke was a decrease in the peripheral blood leucocytes.

Several defects in immune function of these smoke-exposed mice were noted. Humoral immune responses of the mice to intratracheal inoculations of sheep erythrocytes were examined by measuring plaque-forming-cell responses in the mediastinal and cervical lymph nodes, spleen and lungs as well as serum antibody titres. Primary responses in all these organs were ultimately depressed by inhalation of smoke, but plaque-forming cell responses in the lymph nodes and spleen were transiently enhanced soon after the commencement of smoke exposure. Responses of the mice chronically exposed to cigarette smoke to a second intratracheal inoculation of sheep erythrocytes were also smaller than the responses of control mice. Impairment of the primary immune responses could be partially reversed by discontinuing the exposure to cigarette smoke. Inhalation of cigarette smoke produced similar changes in primary humoral immune responses of mice to intraperitoneal inoculations of sheep erythrocytes, but the responses of mice exposed to cigarette smoke, to a second intraperitoneal inoculation of sheep erythrocytes, were almost indistinguishable from the responses of control mice. Serum antibody responses to polyvinylpyrrolidone were not influenced by inhalation of cigarette smoke. The responsiveness of lymphocytes from mice to phytohaemagglutinin was transiently enhanced, but eventually depressed by cigarette smoke inhalation. Experiments showed that mice exposed to cigarette smoke could clear bacteria from blood

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as rapidly as control mice but indicated that phagocytic function in the liver was reduced. Bacteriocidal activity in the lungs of mice chronically exposed to cigarette smoke was impaired. It was also found that growth of established, immunogenic tumour cells in mice was enhanced if the mice were chronically exposed to cigarette smoke.

The effects of cigarette smoke on some isolated elements of the immune system were also studied. Macrophages were very sensitive to acute toxic effects of cigarette smoke and macrophages surviving exposure to smoke had impaired phagocytic activity. However if macrophages, exposed to cigarette smoke, were incubated in the presence of bacteria for several hours their phagocytic activity became greater than the phagocytic activity of control macrophages. Cigarette smoke did not influence haemagglutinating or haemolytic antibody activity *in vitro* but did have some direct inhibitory action on the function of antibody-producing cells.

These experiments show that the inhalation of cigarette smoke could damage immune function. Damage to the immune system may contribute to the aetiology of neoplasia and respiratory infection in cigarette smokers, by diminishing the defence mechanisms of the body against infectious agents and neoplastic cells.

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Except where acknowledged this thesis is of my own composition.

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EXPLANATORY NOTE

This thesis is divided into five chapters. The immunological studies reported in Chapter 4 have been divided into four sections, each with introduction, results and discussion. This presentation was designed to allow a more detailed discussion of experimental results before leading into a general discussion in Chapter 5.

Some of the results have been published* or are in preparation for publication and these have been referred to at appropriate places in the text.

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* (1) Effect of cigarette smoking on primary and secondary humoral responses of mice. W. R. Thomas, P. G. Holt and D. Keast, Nature, 243, 240. (1973).

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(7) Recovery of immune system after cigarette smoking. W. R. Thomas, P. G. Holt and D. Keast, Nature, 248, 358. (1974).

(8) Growth of transplanted tumours in mice after chronic inhalation of fresh cigarette smoke. W. R. Thomas, P. G. Holt, D. Keast, Br. J. Canc., In press.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

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A. Introduction

Anergy of the immune system, as illustrated by the immunodeficiency syndromes (Janeway 1968; Bellanti and Schlegel 1971; Gatti and Good 1971) and the diseases associated with immunosuppressive therapy (Swanson and Schwartz 1967; Hart, Russel and Remington 1969; Hoover and Fraumeni 1973), can have a profound influence on health. Consequently, agents which depress immune function may markedly impair the health of an animal. This study was designed to examine the effect of inhalation of cigarette smoke on immune responses of mice and to consider the results with reference to both the development of experimental models for studying the effects of cigarette smoking and the possible involvement of immunosuppression, induced by cigarette smoke, in the aetiology of some of the diseases associated with cigarette smoking. Several expert committees have published reports on the relationship of cigarette smoking and health (Royal College of Physicians 1962, 1971; United States Public Health Service 1964, 1971), and comprehensive bibliographies of the world literature have been compiled (Larson, Haag and Silvette 1961; Larson and Silvette 1968; Larson and Silvette 1971). Cigarette smoking has been shown to be associated with an increased incidence of cardiovascular disease, neoplasia, respiratory infection, chronic bronchitis and emphysema (Royal College of Physicians 1962, 1972; United States Public Health Service 1964, 1971). This chapter will examine immunological aspects of diseases associated with cigarette smoking and review the literature relating to the influence of cigarette smoking on immunological competence.

B. Immunological aspects of diseases associated with cigarette smoking

(a) Infection

Cigarette smokers have been reported to have increased mortality from influenza infection and pneumonia (United States Public Health Service 1964). The increased respiratory tract infections have not just been related to mortality and chronic respiratory disease; several studies have shown that cigarette smokers in populations of healthy young people have an excessive incidence of acute bacterial and viral respiratory infection (Haynes, Krstulovic and Loomis 1966; Peters and Ferres 1967; Finklea, Sandifer and Smith 1969; Finklea, Hasselblad, Sandifer, Hammer and Lowrimore 1971). A correlation between incidence of upper and lower respiratory tract

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infection and number of cigarettes smoked has been reported (Haynes *et al* 1966). There is evidence that cigarette smokers are more likely to suffer from pulmonary tuberculosis than are nonsmokers. However this is complicated by the relationships between cigarette smoking and alcohol consumption (Royal College of Physicians 1971; United States Public Health Service 1971).

Mice have been shown to have an increased susceptibility to infection, with *Klebsiella pneumoniae* and *Diplococcus pneumoniae*, following the inhalation of cigarette smoke (Spurgash, Ehrlich and Petzold 1968). Chronic and acute exposure of mice, hamsters and squirrel monkeys to nitrogen dioxide, a component of cigarette smoke, decreases their resistance to *Klebsiella pneumoniae* and influenza virus (Ehrlich 1966; Ehrlich, Henry and Fenters 1970; Henry, Findlay, Spangler and Ehrlich 1970). Tissue culture explants of the lungs from guinea pigs exposed to nitrogen dioxide have been found to have a higher incidence of infection with *Pseudomonas aeruginosa* than the explants of normal guinea pig lungs (Richters, Sherwin, Buckley, Balchum and Ivler 1966). Inhalation of inert particles has been shown to be followed by an increase in microflora in the respiratory tract of rodents (Battigelli 1971).

Some early studies did not find an association between cigarette smoking and common respiratory infections (Dowling, Jackson and Inouye 1957; Boake 1958; Heath 1958). These included an experiment involving the infection of volunteers with common cold virus(s) (Dowling *et al* 1957). However more recent studies with large, comparatively uniform populations of young people, and experimental animals, have shown that inhalation of cigarette smoke can increase susceptibility to respiratory tract infection.

Increased respiratory tract infection is found in patients with immunodeficiency disease (Janeway 1968; Bellanti and Schlegel 1971) and patients under immunosuppressive treatment (Hill, Rowlands and Riflind 1964). The mechanisms by which the immune system protects against infectious agents depend on the individual host-parasite relationships but some generalisations have been made (Bellanti 1971; World Health Organization 1973):- Humoral immune mechanisms are important in protection against bacterial infections, particularly bacteria which can be readily phagocytosed and inactivated by macrophages. The humoral immune system also protects against some virus infections, especially against reinfection. Cell-mediated immunity

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is the principal defence against the chronic bacterial infections associated with the ability of the bacteria to survive intracellularly in normal macrophages. It has also been implicated as the main defence against primary acute viral infections and fungal infections. The macrophage is an important element in both humoral and cell-mediated immunity.

As well as the specific immune response there are several nonspecific defence mechanisms against infection (Bellanti 1971). The phagocytic activity of alveolar macrophages and the mucociliary transport mechanism of the respiratory tract are particularly important in relation to respiratory infection (Green 1970; Gross and Detreville 1972).

(b) Neoplasia

Considerable evidence has accumulated indicating that the immune system has a protective effect against neoplasia. This has been the subject of a number of reviews (Klein 1968, 1972; Burnet 1970; Doll and Kinlen 1970; Gold 1970; Keast 1970; Piessens 1970; Smith and Landy 1970; Kaplan 1971; Currie 1972). Immunodeficient patients have about 10,000 times greater chance than age-matched controls of developing malignancies (Gatti and Good 1971). The malignancies are primarily disorders of the reticuloendothelial and lymphoid system so it is possible that the increased malignancy and the immunodeficiency have a relationship other than the failure of the immune system to eliminate malignant cells. However a high incidence of malignant disease not related to the lymphoreticular system also occurs. Immunosuppressed transplant patients also display neoplastic disorders of the lymphoid and reticuloendothelial systems, as well as other types of neoplasia (McKhann 1969; Hoover and Fraumeni 1973). Animals which are immunosuppressed have a greater susceptibility to chemical (Miller, Grant and Roe 1963; Grant and Miller 1965; Johnson 1968) and viral induced carcinogenesis (Miller *et al* 1963; Defendi, Roosa and Koprowski 1964; Grant and Miller 1965; Law 1966; Allison and Law 1968).

Although antibody has been implicated in the protection against some types of animal and human neoplasia (Gorer and Amos 1956; Law 1969; Fefer 1970; Klein 1970; Lewis *et al* 1971), cell-mediated immune reactions appear to be the most important defence against neoplasms (Hellström and Hellström 1970b; Klein 1970; Hellström, Hellström,

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Sjögren and Warner 1971a,b; Smith and Landy 1970). Serum factors which can block the activity of immune cells against tumour cells *in vitro* (Möller 1965; Hellström and Hellström 1970b) as well as other serum factors, which can prevent the blocking, (Hellström and Hellström 1970a,b; Bansal and Sjögren 1971) have been found. Many cancer patients, regardless of their clinical conditions, have cell-mediated immune reactions directed towards their tumour cells (Hellström, Hellström, Sjögren and Warner 1971a) but the presence of blocking or unblocking activity has been related to the progression, regression or recurrence of tumour growth in animals and man (Hellström and Hellström 1970a,b; Hellström, Hellström, Sjögren and Warner 1971b; Hellström, Sjögren, Warner and Hellström 1971). This provides evidence that the immune reactions demonstrated against tumour cells *in vitro* parallel the defence mechanisms occurring against tumour cells *in vivo*. Macrophages appear to be important in tumour immunity. Activated macrophages, which can probably be armed with cytophilic antibody, destroy tumour cells *in vitro* (Evans and Alexander 1970, 1972; Hibbs, Lambert and Remington 1971).

Several authoritative committees have considered the evidence from prospective and retrospective studies on the relationship of cigarette smoking to lung cancer (Royal College of Physicians 1971; United States Public Health Service 1971). It has been concluded that lung cancer is strongly associated with cigarette smoking; the risk of developing cancer increases with the duration of cigarette smoking and the number of cigarettes smoked per day, and is diminished by discontinuing smoking. Cigarette smoking has also been implicated in the cause of increased cancer of the mouth, larynx, oesophagus, bladder and pancreas (Royal College of Physicians 1971; United States Public Health Service 1971). The predominant histological types of respiratory tract cancer found in cigarette smokers has been the squamous cell and oat cell carcinomas (Royal College of Physicians 1971; United States Public Health Service 1971). However several studies have shown that an increased incidence of adenocarcinomas also occurs (Berg 1970; United States Public Health Service 1971; Weiss, Boucot, Seidman and Carnaham 1972). Chronic inhalation of cigarette smoke has been found to increase pulmonary neoplasia in mice (Harris and Negroni 1967; Leuchtenberger and Leuchtenberger 1970a) and dogs (Auerbach, Hammond, Kirman and Garfinkel 1970a). The production of cancer of the larynx by exposing

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hamsters to cigarette smoke has also been reported (Dontenwill 1973). While the neoplasms produced in the mice were adenomas or adenocarcinomas, the neoplasms in the dogs and hamsters were more compatible with the histology of the lung cancer in human cigarette smokers.

Cigarette smoke contains many known carcinogens, one of the principal carcinogens, benz(α) pyrene, being one of the most effective carcinogens known (Wynder and Hoffmann 1964; Stedman 1967). The condensate of cigarette smoke has been shown to produce epidermoid carcinomas and other neoplasms of the skin (Wynder and Hoffmann 1964, 1970; Day 1967) and lungs (Rockey, Speer, Ahn, Thompson and Hirose 1962). Cigarette smoke condensate and fractions of cigarette-smoke condensate can transform cells *in vitro* (Freeman *et al* 1971; Rhim and Huebner 1973). However the concentration of carcinogens in cigarette-smoke condensate can only account for a small proportion of the carcinogenic activity of cigarette smoke (Druckery 1961; Wynder 1961; Wynder and Hoffmann 1964). Because cigarette smokers generally inhale the small quantities of carcinogens several times daily, in a complex mixture of gases and particles, evaluation of the carcinogenic effect of cigarette smoking is difficult. Cigarette-smoke condensate is a more potent carcinogen than would be expected by considering the carcinogenic activity of the individual components (Wynder, Fritz and Furth 1957; Wynder and Hoffmann 1964). This is considered to be due to the synergistic action of weak carcinogens and cocarcinogens (Wynder and Hoffmann 1964; Van Duuren 1971; Sydnor, Allen and Higgens 1972). Uranium miners who smoke cigarettes have an incidence of lung cancer which is indicative of a synergistic action between the inhaled radioactive elements and the cigarette smoke (Archer, Wagoner and Lundin 1973). A similar synergistic effect apparently increases the incidence of lung cancer in asbestos workers who smoke cigarettes (Selikoff, Hammond and Churg 1968). Radioactive elements have also been found in the mainstream of cigarette smoke but their relation to the initiation of neoplasms is obscure (Stedman 1967; Royal College of Physicians 1971; United States Public Health Service 1971).

The carcinogenic and cocarcinogenic activity in the vapour phase of cigarette smoke as well as in the condensate must also be considered. The Leuchtenbergers found that the vapour phase produced more pulmonary tumorigenesis in mice than whole smoke

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(Leuchtenberger and Leuchtenberger 1970a). The vapour phase was also able to produce changes in the DNA metabolism and mitosis of cells in tissue-culture explants from human lung (Leuchtenberger, Leuchtenberger and Schneider 1973; Leuchtenberger, Leuchtenberger, Ritter and Inui 1973) as well as in other types of cells (Leuchtenberger and Leuchtenberger 1969, 1970b). However people smoking filtered cigarettes have been found to have a lower incidence of lung cancer than people smoking unfiltered cigarettes (Royal College of Physicians 1971; United States Public Health Service 1971; Wynder 1972).

The cocarcinogenic activity of cigarette smoke could be directed to the prospective malignant cell or could act on mechanisms controlling the growth of malignant cells. Alterations in the endocrine system have been implicated in the development of lung cancer (Rao 1970). Another regulatory influence is the immune system. There is substantial evidence indicating that the carcinogenic aromatic hydrocarbons, a group of carcinogens which are present in cigarette smoke (Wynder and Hoffmann 1964; Stedman 1967), exert much of their carcinogenic action by an immunosuppressive effect (Rubin 1960; Linder 1962; Prehn 1963; Stjernswärd 1965, 1966a,b, 1967; Ball 1970). Some data has shown that closely related but non-carcinogenic hydrocarbons do not have an immunosuppressive action (Stjernswärd 1966a, 1967) and strains of mice that are refractory to the carcinogenic action of the hydrocarbons are not susceptible to their immunosuppressive effect (Stutman 1969).

(c) Chronic bronchitis

Cigarette smoking has been associated with both an increased incidence and risk of dying from chronic bronchitis (United States Public Health Service 1971; Royal College of Physicians 1971). Cigarette-smoke exposure has produced chronic bronchitis in dogs (Auerbach *et al* 1967) rats (Lamb and Reid 1969) and mice (Leuchtenberger *et al* 1963). A relationship between the immune system and chronic bronchitis lies in the involvement of respiratory tract infection in the pathogenesis of the disease. Although respiratory tract infection is probably not the primary cause of most cases of chronic bronchitis (Chodosh and Segal 1964; Rodman and Sterling 1969), there is evidence that bacterial and viral infections produce exacerbation of the disease (Stuart-Harris 1965; Green 1970).

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Antibiotics have decreased the production of mucous by patients with chronic bronchitis (Edwards, Chanley, Keal and Fear 1963; Chodash and Segal 1964; Oswald 1964).

(d) Emphysema

Emphysema has been associated with both cigarette smoking and chronic bronchitis (Thurlback and Angus 1963; United States Public Health Service 1964; Rodman and Sterling 1969; Royal College of Physicians 1971). However both emphysema and chronic bronchitis can occur independently (Thurlback and Angus 1963; Rodman and Sterling 1969). Lesions similar to emphysema have been produced by chronically exposing animals to cigarette smoke (Rockey *et al* 1962; Hernandez, Anderson and Foraber 1966; Auerbach, Hammond, Kirman and Garfinkel 1970b; Hammond, Auerbach, Kirman and Garfinkel 1970). It has been suggested that emphysema and chronic-respiratory-obstructive disease result from an impairment of immune function in the lower respiratory tract (United States Public Health Service 1964; Green 1970; Kilburn 1973). There is evidence that emphysema results from the action of proteolytic enzymes from leucocytes on the architecture of the lung (Mario, Mass, Meranze and Wainbaum 1971; Mittman 1972; Lieberman 1973). The inhalation of cigarette smoke has been found to produce an increase in the number of macrophages which can be recovered from the lungs by endobronchial lavage, in humans (Harris, Swenson and Johnson 1970a) and animals (Rylander 1971a). As cigarette smoke can kill alveolar macrophages *in vitro* and *in vivo* (Holt and Keast 1973a,b) smoking may also have a role in releasing the enzymes from the macrophages.

Factors other than cigarette smoking have been shown to be important in the aetiology of emphysema (Rodman and Sterling 1969). In particular, a deficiency of serum alpha-1-antitrypsin activity predisposes people to developing emphysema (Laurell and Eriksson 1963; Briscoe, Kueppers, Davis and Bearn 1966). However it has been reported that cigarette smoking further increases the prevalence of emphysema in people with the alpha-1-antitrypsin deficiency (Lieberman 1969; Mittman, Lieberman, Manasso and Miranda 1971).

(e) Cardiovascular disease

A large number of reports have associated cardiovascular disease with immunological hypersensitivity reactions to cigarette

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smoke or cigarette-smoke components. These have been the subject of recent reviews (Harkavy 1968; Swartz 1971) and will not be considered in detail.

Macrophages are thought to have a significant role in the metabolism of lipids (Stuart 1970). As high levels of serum lipid have been associated with arterial disease (French 1970), an impairment of the ability of macrophages to metabolise lipid may contribute to the development of cardiovascular disease.

C. Immunity and the respiratory system

A consideration of immune responses, in the respiratory tract and response to inhaled immunogens is central to this thesis, because: (i) It is normal to constantly inhale air which can contain infectious agents, carcinogens and other toxic elements (World Health Organization 1972); (ii) The major toxic action of cigarette smoke inhalation could reasonably be expected to occur in the respiratory tract; (iii) The infection and neoplasia associated with cigarette smoking are principally found in the respiratory tract.

(a) Immunity against inhaled agents

The architecture of air passages promotes the deposition of inhaled particles in the upper respiratory tract where they can be cleared by the mucociliary transport of the tracheobronchial system (Green 1970). Foreign material deposited beyond tracheobronchial clearance mechanisms reach the phagocytic and lymphatic system of the lungs (Stuart 1970; Green 1970; Bowden 1971; Gross and Detreville 1972). Unphagocytosed material can also reach the lymphatic system (Schneeberger-Keely and Karnovsky 1968). The alveolar macrophages can rapidly phagocytose and inactivate bacteria and have an important function in maintaining sterility of the pulmonary system (Green and Kass 1964a,b).

Immunogens entering the respiratory system can induce humoral or cell-mediated immune reactions (Tomasi and Bienenstock 1968; Waldman and Henney 1971; Mackaness 1971). These responses can be initiated locally in the respiratory tract (Tomasi and Bienstock 1968; Waldman and Henney 1971). The local humoral immune response consists primarily of the production of secretory IgA antibody

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(Tomasi and Bienstock 1968) but other classes of antibody are also produced locally (Waldmann and Henney 1971). Antibody producing cells have been found in the lamina proprium of the bronchi (Martinez, Tello, Braun and Blanc, 1968; Fernald, Clyde and Bienenstock 1972), and in the endobronchial lavages of locally immunized animals (Holub and Hauser 1969; Ford and Kuhn 1973a,b). Inhaled immunogens can also induce a systemic humoral immune response (Artenstein, Bellanti and Buescher 1964; Cate, Rossen, Douglas, Butler and Couch 1966; Lieberman *et al* 1971). There are several studies reporting that antibody in the respiratory secretions is a better indication of protection against viral infection than serum antibody (Fazekas de St Groth and Donnelly 1950; Cate *et al* 1966; Smith, Purcell, Bellanti and Chanock 1966; Chanock 1970) but some studies have shown that the protection from virus infection is more closely related to the serum antibody titre (Slepushkin *et al* 1971). The effectiveness of the secretory immune system in the protection against infection can be illustrated by the high incidence of respiratory infection in patients with selective IgA deficiency (Schwarz and Buckley 1971; Polmar *et al* 1972). Immunosuppressive treatment has been shown to produce bacteraemias by allowing the growth of bacteria from the respiratory tract (Wensinck 1961a,b).

Immunogens administered to the respiratory tract can elicit both local and systemic cell mediated immune responses (Henney and Waldman 1970; Truitt and Mackaness 1971; Waldman, Spencer and Johnson 1972). Human alveolar macrophages are responsive to macrophage migration inhibition factor (MIF) (Bartfeld and Atoynatan 1970; Warr and Martin 1973a) which indicates that they can be an effector cell in cell-mediated immune reactions.

(b) Respiratory tract tumour immunity

The potential defence mechanisms of the respiratory system against both carcinogens and neoplastic cells should be considered. This also includes defences against oncogenic viruses. Alveolar macrophages ingest material inhaled in the cigarette smoke (Mellors 1958; Leuchtenberger, Leuchtenberger, Zebrun and Schaffer 1960; United States Public Health Service 1964; Pratt, Finley, Smith and Ladman 1969) and can metabolise carcinogens (Tomingas *et al* 1971). Enzymes for the metabolism of aromatic hydrocarbons are elevated in animals exposed to cigarette smoke (Welch, Loh and Conney 1971;

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Okamoto, Chan and So 1972; Holt and Keast 1973c). However the metabolism of hydrocarbons may have two opposing functions: (i) the detoxification of carcinogens; (ii) the production of highly carcinogenic intermediates during the detoxification (Alexandrov and Frayssinet 1972; Marquardt *et al* 1972).

Mice with pulmonary neoplasia can elicit an immune response against the neoplastic cells (Colnaghi, Menard and Della Porta 1971). In one study the ability of urethane to produce lung tumours in mice was proportional to the degree of immunosuppression it induced (Menard, Colnaghi and Cornalba 1971). Furthermore the immunogenicity of the tumours was increased with increased immunosuppression by the urethane. Richters, Sherwin and Richters (1971) observed the interactions of lymphocytes with cells from resected lung cancers and concluded that a defective lymphocyte-tumour cell interaction occurred in lung neoplasia. An increase of IgA and IgG in the bronchial secretions of patients with lung cancer has been reported (DeCosse, Mandel and Dvorak 1971). The levels of immunoglobulin detected were considerably higher than the levels occurring in bacterial inflammation. Selective IgA deficiency has not generally been associated with increased neoplasia (Gatti and Good 1971; Hong and Ammann 1972) but neoplasms have been reported in patients with selective IgA deficiency (Gatti and Good 1971). Patients developing empyema after the surgical removal of lung carcinoma have a considerably increased five-year-survival-rate. It is thought that the bacterial infection non-specifically enhances the tumouricidal activity of the immune system (Ruckdeschel, Codish, Stranahan and McKneally 1972).

D. Cigarette smoking and immunity

The interaction of cigarette smoking and the immune system has been reviewed previously (Silvette, Larson and Haag 1957; Larson, Haag and Silvette 1961; Harkavy 1968; Larson and Silvette 1968, 1971; Swartz 1971), but the principal consideration was directed towards the hypersensitivity to cigarette smoke and its relationship to cardiovascular disease. In this section the effect of cigarette smoking on immunological competence will be reviewed.

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(a) Peripheral blood leucocytes

Human cigarette smokers have been reported to have an increased number of peripheral blood leucocytes (Howell 1970; Corre, Lellouch and Schwartz 1971; Friedman *et al* 1973). The increase was found to be related to the amount of cigarette smoke inhaled (Corre *et al* 1971; Friedman *et al* 1973) and represented an increase in all types of leucocytes (Corre *et al* 1971). Friedman *et al* (1973) were able to demonstrate that leucocyte counts increase in people after they begin cigarette smoking and decrease in people who discontinue smoking. Corre *et al* (1971) concluded that the increase in leucocytes was not related to the incidence of chronic bronchitis but the results of Friedman *et al* (1973) indicate that chronic bronchitis was a factor in the leucocytosis. Examination of leucocytes in animals chronically exposed to cigarette smoke has produced variable results. No alterations in the number of peripheral blood leucocytes were found in hamsters exposed to cigarette smoke (Reckzeh and Dontenwill 1970; Dontenwill *et al* 1973). Reece and Ball (1960) exposed dogs to cigarette smoke for 15 weeks and found an increase in blood monocytes but because of a decrease in the concentration of neutrophils and lymphocytes the total white blood cell count was decreased.

(b) Pulmonary leucocytes

As judged by endobronchial lavage, cigarette smokers have more macrophages in their lungs than nonsmokers (Pratt *et al* 1969; Harris *et al* 1970a). This has also been found in guinea pigs (Rylander 1971a) and mice (Holt and Keast 1973a) chronically exposed to cigarette smoke. Mellors (1958) observed that there are more alveolar macrophages in sections of lungs from rats exposed to cigarette smoke than in the lungs of control rats. Flint, Maxwell and Renzetti (1971) reported that the number of polymorphonuclear leucocytes increased in the lungs of guinea pigs while the number of macrophages was unaltered by exposure to cigarette smoke. A transient increase in the number of lymphocytes in the lungs of mice exposed to cigarette smoke has also been found (Holt and Keast 1973a).

(c) Metabolism and morphology of leucocytes exposed to cigarette smoke *in vivo*

Alveolar macrophages from human cigarette smokers have a

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larger endoplasmic reticulum and Golgi apparatus than macrophages of nonsmokers (Harris *et al* 1971a,b) indicating that the macrophages from smokers have a higher rate of protein synthesis. Cytoplasmic inclusions have also been found in macrophages from human cigarette smokers (Pratt *et al* 1969; Harris *et al* 1971a; Pratt, Smith, Ladman and Finley 1971; Martin 1973). The inclusions are probably phagolysosomes which can contain needle like structures (Pratt *et al* 1971). The alveolar macrophages from rabbits exposed to cigarette smoke for 20 days have less fenistrae than control macrophages, when examined with the scanning electron microscope (Aranyi 1972).

Cultured alveolar macrophages from cigarette smokers have been reported to exhibit a higher oxidative metabolism than the macrophages from nonsmokers (Harris *et al* 1971a,b) but histochemical studies have shown a decrease in the oxidoreductive enzymes of the alveolar macrophages from smokers (Roque and Pickren 1968). The decrease in enzyme activity was proportional to the amount of fluorescent material, from cigarette smoke, stored by the macrophage. This histochemical study also reported a decrease in acid hydrolase activity (Roque and Pickren 1968) which is at variance with the results of Martin (1973) which described highly elevated activities of acid hydrolase and other hydrolytic enzymes. Macrophages lavaged from the lungs of mice exposed to cigarette smoke have been shown to have a higher rate of RNA metabolism than macrophages from control mice (Holt and Keast 1973b).

Alveolar macrophages from cigarette smokers have an increased ability to adhere to glass (Mann, Cohen, Finley and Ladman 1971). Acute cigarette smoke exposure has been reported to decrease the locomotion and oxidative metabolism of oral leucocytes (Eichel and Shahrik 1969). The alveolar macrophages from cigarette smokers are able to migrate from capillary tubes, faster than the macrophages from nonsmokers (Warr and Martin 1973). The migration of the macrophages from smokers was not inhibited by MIF (Warr and Martin 1973). The inhalation of cigarette smoke has been shown to kill the macrophages in the lungs of mice but some resistance to this acute toxic effect was developed after chronic inhalation of cigarette smoke (Holt and Keast 1973a).

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(d) Phagocytosis after exposure to cigarette smoke *in vivo*

No difference has been found between the phagocytic activity of macrophages cultured *in vitro* from human cigarette smokers or non-smokers (Cohen and Cline 1971; Harris *et al* 1971a; Mann *et al* 1971). However guinea pigs exposed to cigarette smoke for four weeks had a depressed pulmonary phagocytic activity (Rylander 1971b) even though cigarette smoke increased the number of macrophages in their lungs (Rylander 1971a). It has also been reported that after two years exposure to cigarette smoke the phagocytic mechanism in the lungs of mice appeared to be overwhelmed by cigarette smoke deposits; large areas of the lung parenchyma were flooded with pigment in the absense of macrophages (United States Public Health Service 1964). Similar overloading of macrophages with particulate matter has been reported (Rylander 1969; Bowden 1971). Lentz and DiLuzio (1973) have reported that the alveolar macrophages of rabbits exposed to cigarette smoke have a normal phagocytic activity but the smoke exposure does decrease the activity of a substance in the lungs which promotes phagocytosis.

The phagocytosis of particles in the lungs can be inhibited by acute exposure to cigarette smoke (Sanders *et al* 1971). The elimination of viable bacteria from the lungs of mice is also inhibited by acute exposure to cigarette smoke (Laurenzi, Guarneri, Carey and Endriga 1963; Laurenzi, Guarneri, Endriga and Carey 1963; Laurenzi, Guarneri and Endriga 1965). This elimination of viable bacteria, which is primarily due to the action of alveolar macrophages (Green and Kass 1964a,b), was not influenced by exposure to cigarette smoke in a strain of mice with a rapid rate of bacterial elimination (Laurenzi *et al* 1963). In order for exposure to cigarette smoke to inhibit the elimination of viable bacteria from the lungs it was found that intestinal bacteria or an intraperitoneal inoculation of endotoxin was required (Laurenzi, Guarneri and Engriga 1965). Chronic and acute exposure of animals to nitrogen dioxide, a component of cigarette smoke, also decreases phagocytic activity and the rate of elimination of viable bacteria from their lungs (Ehrlich, Henry and Fenters 1970; Acton, Myrvik and Winston-Salem 1972; Goldstein, Eagle and Hoeprich 1973).

The phagocytic activity of peripheral blood leucocytes from smokers has been reported to be less than the activity of the

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leucocytes from nonsmokers (Kadlubowski (1950) cited by Larson *et al* 1971). The same results were obtained with rabbits after acute and chronic exposures to cigarette smoke (Bruni (1931) cited by Larson *et al* 1971). This was not caused by a decrease in the opsonic activity in the serum. Nicol and Cordingley (1964) have shown that the chronic administration of nicotine to mice does not affect their rate of clearance of particles from blood.

(e) Metabolism of macrophages exposed to cigarette smoke *in vitro*

During experiments in which lung explants from foetal mice were exposed to cigarette smoke for several weeks in culture, Leuchtenberger and Leuchtenberger (1969, 1970b) observed that macrophages were very susceptible to the toxic effects of cigarette smoke. They reported a decrease in both the number of macrophages and the RNA systhesis of the macrophages, as judged by histochemical techniques. Monolayer cultures of alveolar and peritoneal macrophages have been found to be more susceptible to the toxicity of cigarette smoke than either fibroblastic or epithelioid cells from lungs of foetal mice (Holt and Keast 1973b; Holt, Bartholomaeus and Keast 1974). Macrophages which survived the exposure to cigarette smoke had a higher rate of RNA synthesis than control cultures 30 minutes after the exposure, but their RNA synthesis was decreased 24 hours after the exposure. Macrophages with a high basal rate of RNA synthesis were less susceptible to the toxic effects of the cigarette smoke (Holt and Keast 1973b). Yeager (1969) and Holt and Keast (1973d) have shown that the protein synthesis of macrophages is decreased by the acute exposure to solutions of cigarette smoke but macrophages repeatedly exposed to low levels of smoke have a marked increase in RNA and protein synthesis (Holt and Keast 1973d). Cigarette-smoke solutions have been shown to decrease the oxygen consumption of macrophages (York *et al* 1973) and Lentz and DiLuzio (1972, 1973) have reported that cigarette smoke depresses the oxidation of glucose by macrophages. Other experiments have shown that the activity of enzymes isolated from alveolar macrophages can be inhibited by solutions of cigarette smoke (Powell and Green 1971). Nicotine has also been shown to decrease the oxygen consumption and adenosine triphosphatase activity of alveolar macrophages (Meyer, Cross, Ibram and Mustafa 1971).

Thus in general the results of the experiments *in vitro*

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show that cigarette smoke has an acute toxic effect on macrophages but prolonged cigarette smoke exposure can produce macrophages with an elevated metabolic activity. This concurs with the observations of Harris *et al* (1971a,b) who examined the alveolar macrophages from cigarette smokers.

(f) Phagocytosis after exposure to cigarette smoke *in vitro*

Green and Carolin (1967) have shown that cigarette smoke could inhibit the killing of *Staphylococcus albus* by rabbit alveolar macrophages. The active component was in the vapour phase of the cigarette smoke, but nicotine, acetaldehyde, formaldehyde and cyanide, in doses comparable to their concentration in cigarette smoke did not affect the macrophages. Subsequently it was found that glutathione or cysteine could protect the macrophages (Green 1968). If the inactivation of bacteria was measured in the absence of serum there was a 30% reduction in killing activity, but the bacteriocidal activity was either not affected or slightly increased by cigarette smoke. In the presence of serum, however almost 100% of the phagocytic activity of the macrophages could be decreased by an exposure to cigarette smoke. Lentz and DiLuzio (1972, 1973) reported that the killing and degradative mechanisms of alveolar macrophages were not impaired by cigarette smoke but the ingestion of bacteria and other material was inhibited. They have also reported that cigarette smoke did not impair phagocytosis by liver macrophages. The phagocytic activity of alveolar macrophages can be inhibited by the exposure to nitrogen dioxide *in vitro* (Vassallo *et al* 1973).

(g) Humoral immunity

Experiments with animals have shown that the inhalation of cigarette smoke can decrease serum antibody titres. Donzelli (1933) (cited by Silvette *et al* 1957) found that inhalation of cigarette smoke decreased antibody titres of rabbits immunized with typhoid. From a recent study, Esber, Menninger, Bogden and Mason (1973) reported that both primary and secondary antibody responses of mice to sheep erythrocytes were inhibited by short term cigarette-smoke exposure. The inhibition of antibody response could be detected two weeks after exposing the mice to cigarette smoke for one week.

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A survey of serum antibody titres, to influenza virus, of military cadets has shown that cigarette smokers initially responded to infection or vaccination as well as nonsmokers (Finklea, Hasselblad, Riggan, Nelson, Hammer and Newill 1971). However the persistance of hemagglutinating antibody to influenza antigen was decreased in cigarette smokers when compared to nonsmokers. It has also been reported that smokers have a depressed level of natural agglutinins of sheep erythrocytes (Fletcher, Sumney, Langkamp and Platt 1968), but the ages and health of the smokers were different from those of the nonsmokers examined. Another investigation, however, found cigarette smokers had a higher titre of antibody (precipitin) to *Haemophilus influenzae* than nonsmokers (May, Peto, Tinker and Fletcher 1973). It was concluded from the results of a multiple regression analysis that the stimulation of these serum antibody levels was not dependent on respiratory infection. This analysis did not consider subclinical infections but in any case stimulation or inhibition of serum antibody responses to respiratory infection by cigarette smoking may only reflect an abnormality of defense mechanisms in the respiratory tract. Human cigarette smokers have also been found to have increased serum C-reactive protein levels and an abnormal seroflocculant for ethyl choledienate (Heiskell, Miller, Aldrich and Carpenter 1962).

Experiments performed *in vitro* have shown that nicotine or aqueous extracts from cigarette smoke rapidly and irreversibly inhibited an early event in the induction of antibody from immunocompetent cells by antigen (Roszman and Rogers 1973). The concentration of smoke solution used in these experiments did not influence the viability of the lymphocytes.

Components of cigarette smoke have been shown to both inhibit and stimulate antibody responses. Guardasciope (1942) (cited by Silvette *et al* 1957) found that nicotine could decrease antibody responses of rabbits to ox erythrocytes. Chronic exposure to nitrogen dioxide increased the serum neutralising antibody titres to influenza infection in squirrel monkeys (Fenters, Findlay, Port, Ehrlich and Coffin 1973). Inhalation of carbon particles caused a transient stimulation of the antibody-forming cell response in mediastinal lymph nodes of mice to an aerosol of *Escherichia coli* (Zarkower 1972a,b). The response in these nodes was eventually decreased as well

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as in the spleen where no transient enhancement occurred.

(h) Cell-mediated immunity

There is little information relevant to cell-mediated immunity in cigarette smokers. The vapour phase of cigarette smoke has been shown to decrease the number of human peripheral blood lymphocytes which transform in the presence of phytohemagglutinin (PHA) (Desplaces, Charneine and Izard 1971). This suggests the possibility that the activation of thymus-dependent lymphocytes, by antigens, may be impaired by cigarette smoke (Rodey and Good 1969; Adler *et al* 1970).

The recent report that alveolar macrophages of cigarette smokers will not respond to MIF indicates that an important defect of cell-mediated immunity may exist in the lungs of cigarette smokers (Warr and Martin 1973).

(i) Tracheobronchial clearance

Although the mucociliary clearance mechanism of the bronchi and trachea is not strictly a part of the immune system it constitutes an important pulmonary defense mechanism (Green 1970; Gross and Detreville 1972). Many reports have shown that the ciliary action of the respiratory epithelium of several animals can be inhibited by cigarette smoke (United States Public Health Service 1964). However the question of whether the clearance of inhaled particles from the lungs of cigarette smokers is depressed is unresolved. Rylander (1971) has shown that the chronic exposure of guinea pigs to cigarette smoke depresses the tracheobronchial clearance of inhaled bacteria. The clearance of inhaled particles from donkeys chronically exposed to cigarette smoke is also depressed (Albert, Spiegelman, Shatsky and Lippmann 1969) but La Belle, Bevilacqua and Brieger (1966) did not find an impairment of tracheobronchial clearance in rabbits exposed to cigarette smoke over a period of fifteen days. Experiments measuring the clearance of radiolabelled particles inhaled by humans have been conducted. Cigarette smoking has been shown to stimulate the tracheobronchial clearance of cigarette smokers (Camner, Philipson and Arvidsson 1971). Albert, Lippmann and Briscoe (1969) reported that some cigarette smokers had impaired clearance mechanisms but Thomson and Pavia (1973), who conducted a larger study, did not find an impairment in cigarette smokers. It has been reported that the

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tracheobronchial clearance of smokers improves after discontinuing smoking (Camner, Philipson and Arvidsson 1973). Camner and Philipson (1972) also found that in five out of ten monozygotic twins with discordant smoking habits, the smoking twin had a slower clearance of inhaled particles. The evidence indicating that tracheobronchial clearance is impaired in cigarette smokers is supported by morphological observations showing alterations and replacement of ciliated cells in the respiratory epithelium of cigarette smokers (Chang 1957; Auerbach *et al* 1962). It is probable that the impairment of the clearance mechanisms in an isolated area of the tracheobronchial system can have a considerable influence on health.

E. Concluding remarks

It is apparent from the review of the literature that information concerning immunity in cigarette smokers is fragmentary and often controversial but there are suggestions that humoral and cell-mediated immunity may be impaired in cigarette smokers. Research into the effect of cigarette smoking on the immune system appears to have been mainly directed towards studying the effect of cigarette smoke on macrophages. However the bacteriocidal and phagocytic capacity of the macrophages in the lungs of human cigarette smokers is not known.

While the diseases associated with cigarette smoking can be considerably reduced if people discontinued smoking there are indications that a substantial proportion of cigarette smokers will continue to smoke and that a large number of people will adopt the habit (Workship of the Second World Conference on Smoking and Health 1972). It has been recognised that as well as discouraging cigarette smoking there is a need to develop cigarettes which are less injurious to health and methods of preventing the diseases occurring in cigarette smokers. The latter possibilities require the identification of the harmful component(s) of cigarette smoke and their mechanism of action.

The experimental studies in the following chapters describe investigations into the immunological competence of mice chronically exposed to cigarette smoke. Humoral and cell-mediated immunity and phagocytic function have been examined. Some studies on the action

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of cigarette smoke on components of the immune system *in vitro* have been described. The relevance of these investigations to the concept that immunosuppression, produced by the inhalation of cigarette smoke, may be involved in the aetiology of the disease patterns found in human cigarette smokers and in animals exposed to cigarette smoke, has been discussed.

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CHAPTER 2

MATERIALS AND METHODS

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A. Exposure to cigarette smoke

(a) Exposure of mice

Mice were exposed to cigarette smoke in a Hamburg II inhalation apparatus (Heinr Borgwaldt; West Germany). The apparatus (Fig. 1) has been developed and described by Dontenwill (1970). The noses and mouths of the mice were admitted to the inhalation chamber which was supplied with a fresh mixture of smoke diluted in air at a ratio of 1:7 (smoke:air) every other second. The cigarette smoke was supplied by a 35ml "puff" from an individual cigarette. The mice were exposed to 30 cigarettes per day in cycles in which a puff was taken from each cigarette (7 minutes continuous cigarette smoke exposure). King sized filter cigarettes supplied by the Australian Tobacco Research Foundation were used for all studies. Except where otherwise specified mice were 6 weeks old at the commencement of their exposure period.

Because of the facilities available, control mice were not placed in the smoking apparatus without cigarettes. Other studies have shown that placing mice in an inhalation apparatus for prolonged periods did not influence body-weight (Leuchtenberger and Leuchtenberger 1970a) and similar handling of animals, 3 times a day, over a period of 2 weeks did not depress the immune response (Esber *et al* 1973). Judging by the movements of the mice it was observed that the actual exposure to smoke produced the most stress and that mice became accustomed to the smoke after several weeks of exposure. Accordingly experiments were designed to differentiate between the effects of chronic exposure and the effect of short-term exposure, where stress reactions were most expected.

(b) *In vitro* exposure to cigarette smoke

Cell cultures were exposed to cigarette smoke in a perspex chamber (Fig. 2), designed to produce fresh cigarette smoke-air mixtures from one cigarette in a ratio of 1:7 (smoke:air). The chamber was designed and reported by Holt and Keast (1973b). The exposure cylinder (F) was 27cm long and 5.8cm in diameter. In order to disperse the cigarette smoke a wire coil was placed in the outlet from the cigarette holder (D). The tissue cultures in 35mm petri dishes were placed in position (E), which was 15cm from the

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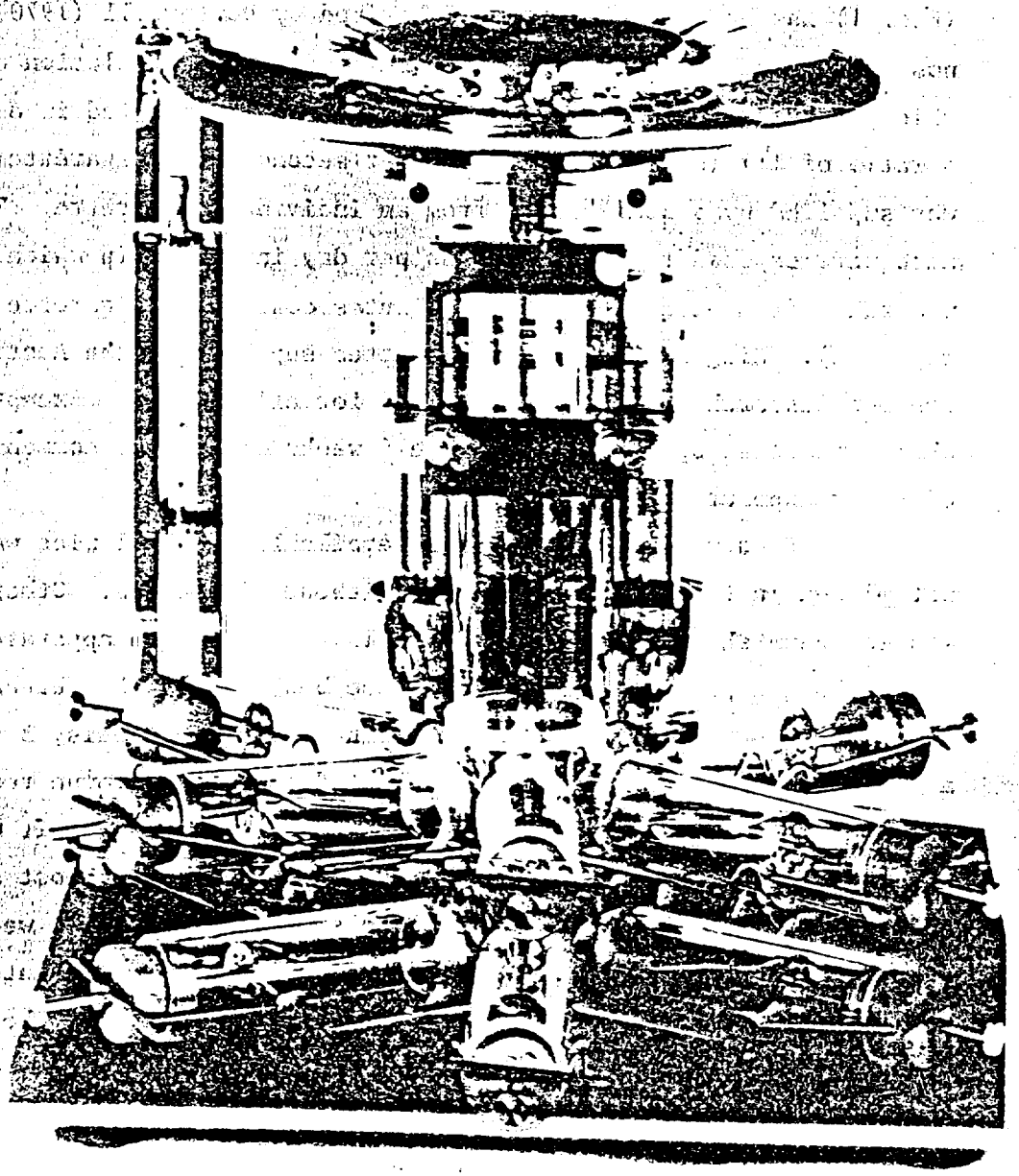

FIGURE 1 Hamburg II smoking machine.

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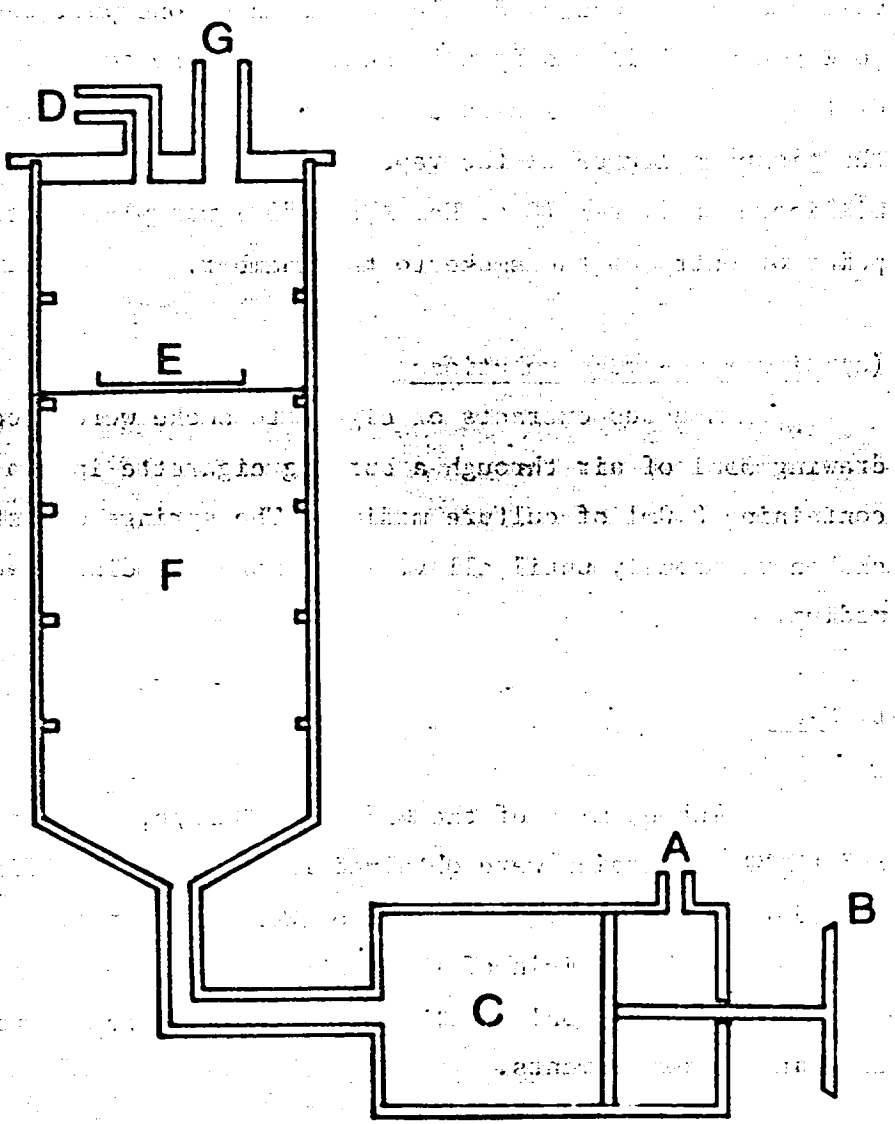

FIGURE 2 Tissue culture smoking machine: vacuum line (A); plunger (B); syringe (volume at position shown (100ml) is 1/7 volume of exposure chamber) (C); cigarette holder (D); tissue culture dish (35mm) 8cm from smoke inlet (E); perspex smoke chamber (30cm in length) (F); outlet to atmosphere (G). To introduce a puff of smoke, outlet G is blocked and plunger (B) is fully withdrawn to position shown. After 2 seconds, the cigarette is removed from D and the plunger (B) is fully withdrawn. The smoke is rapidly cleared from the smoke chamber (F) via the vacuum line (A). After 58 seconds the tissue culture dish is removed from the chamber or when required the cycle repeated.

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entry of the cigarette smoke. In order to produce the smoke a puff volume of 100ml was used. The duration of one puff was standardised to 2 seconds followed by a 58 second exposure to air. A description of the operation is included in the legend of Fig. 2. To expose the tissue cultures to the vapour phase a millipore filter (45μ) and millipore prefilter (Cat. No. AP2004700) was placed across the point of entry of the smoke to the chamber.

(c) Cigarette-smoke solutions

Aqueous extracts of cigarette smoke were produced by drawing 35ml of air through a burning cigarette into a 50ml syringe containing 8.0ml of culture medium. The syringe was stoppered and shaken vigorously until all visible smoke was dispersed in the medium.

B. Mice

Inbred mice of the C57Black, BALB/c, Simpson, C3H, DK Ginger and DK Black strains were obtained from the Perth Medical Centre animal breeding unit (University of Western Australia, Perth, Western Australia). The origin of these strains have been described by Cox and Keast (1973). Unless otherwise specified female mice were used in all experiments.

C. Body weights, organ weights and food consumption

The moist weight of the food and organs and the body weight were determined with the use of a top loading balance (Mettler Model P160, Greifensee - Zurich, Switzerland).

D. Carboxyhaemoglobin (HbCO) estimation

The method for determining the HbCO was adapted from Salt (1951). The percentage haemoglobin not reducable by sodium dithionate was used as a measure of the % HbCO. 50μl of blood were withdrawn from the retro-orbital venous plexus of mice and added to 6ml of 0.1% ammonia solution. The optical density at 600nm was determined by a spectrophotometer (SP 800, Unicam Instruments, Cambridge, England), (R_1). 0.2ml of freshly prepared 10% sodium dithionate was added and.

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the optical density at 600nm measured (R_2). The value $R_2 - R_1$ was calculated for each mouse. As inbred mice, with little variation in haemoglobin concentration, (See tables 5 - 8), were used the %HbCO was calculated:

$$\%HbCO = 100 - \frac{R_2 - R_1 \text{ (test)}}{R_2 - R_1 \text{ (control)}}$$

Groups of 5 test and age-matched controls were used for each determination.

E. Haematology

Blood was collected from the retro-orbital venous plexus of mice in 20µl capillary tubes, (Microcaps, Drummond Scientific Co., Bromall, Philadelphia, U.S.A.), suspended in 10ml of isoton (Nicol and Davis 1971) and haematological data was determined using a Coulter Counter (Model S) (Coulter Electronics Inc., Hialeah, Florida U.S.A.) operated by Mrs. L. Zosky, Division of Haematology, Repatriation Hospital, Perth, Australia). Differential leucocyte counts were performed with May-Grünwald-Geimsa stained blood smears by methods described by Dacie and Lewis (1968).

F. Humoral immune responses to sheep erythrocytes

Sheep erythrocytes (SRBC) were collected in Alsever's solution and stored at $4^{\circ}C$ for periods of up to one week. All SRBC preparations were washed x 3 in phosphate buffered saline (PBS) before use.

(a) Inoculations

Mice were inoculated intravenously into the tail vein or intraperitoneally with 10^8 SRBC in 0.1ml of PBS. For intratracheal inoculations (Thomas, Holt and Keast 1974a) mice were anaesthetised with pentobarbitone sodium (Nembutal, Abbott Laboratories, Australia), and a blunt 19 gauge needle inserted under the epiglottis and almost to the base of the trachea where 10^8 SRBC in 0.01ml of PBS were introduced using an Alga micrometer syringe (Burroughs Wellcome and Co., London, England).

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(b) Plaque-forming cell (pfc) determination

(i) Cell preparations

Mice were exsanguinated and the blood cells allowed to sediment in a final concentration of 0.6% dextran 110 (Glaxo Intradex NCIB 8710) containing 7IU heparin/ml. After one hour the buffy coat was collected and washed twice in growth medium (Eagle's minimal essential, Grand Island Biological Co., F15). The cells were then suspended in 0.2ml of medium. The mediastinal and cervical lymph nodes were removed and pooled, except where indicated. The nomenclature concerning the lymph nodes has been adapted from Dunn (1954). The lungs, spleen and lymph nodes were washed in PBS, then finely minced and suspended in medium. The suspensions were filtered through nylon wool and then washed. The spleen cells were suspended in 2.0ml, and the lymph node and lung in 0.2ml of growth medium. The concentrations of leucocytes obtained from spleen and lymph nodes were about 10^8 and 10^7 cells/ml respectively. Lymphocytes were the predominant type of cell in both preparations. About $5x10^6$ cells/ml could be obtained in the preparations of lung cells. These were 80% - 90% lymphocytes with the remainder consisting of macrophages. Few epithelioid cells were present.

(ii) Haemolytic-plaque assay

The haemolytic-plaque assays were performed in duplicate. 0.02ml aliquots of cell suspension, guinea pig complement, 8%SRBC suspension in PBS and either PBS or a suitable concentration of rabbit anti-mouse globulins were mixed and placed in the chambers similar to those described by Cunningham and Szenburg, (1968). The anti-mouse globulins antiserum was a gift from K. C. Cox (Department of Microbiology, University of Western Australia). The chambers were incubated at 37°C for 30 minutes before counting the plaques. The indirect pfc values were obtained by subtracting the number of plaques developing without the anti-mouse globulins from the plaques developing with this reagent.

(c) Antibody titres

Antibody assays were performed in microtiter plates (Microtiter trays 220-25A, Cooke Engineering Company, Alexandria Virginia, U.S.A.). Haemagglutinating antibody was determined by serially diluting 0.02ml of serum in an equal volume of PBS containing 1% normal rabbit serum. For the reaction 0.02ml of 0.25% SRBC

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in PBS was added to the dilutions. Haemolytic antibody was measured in the same manner using PBS as a diluent and adding 0.02ml of guinea pig serum as a source of complement activity, and 0.02ml of 1% SRBC in PBS to each dilution. The highest dilutions showing complete lysis or definite agglutination were read after 1 hour at 37°C.

G. Antibody response to Polyvinylpyrrolidone (PVP)

Mice were inoculated with 0.2µg of PVP-360 (MW 360,000) (Sigma, St. Louis, Missouri U.S.A.), in 0.1ml of PBS into the tail vein. After one week the serum antibody titre was measured by indirect haemolysis of PVP coated SRBC as described by Andersson and Blomgren (1971) and Andersson (1969). To couple the SRBC and PVP, the SRBC were washed x 3 in PBS and resuspended to 10%. To 10ml of the 10% SRBC, 0.05ml of a 0.01g/ml solution of tannic acid in PBS was added and the mixture incubated for 10 minutes at ambient temperature. The SRBC were then washed x 2 in PBS and resuspended in 10ml and then 0.1ml of 0.01g/ml of PVP (MW 24,500) was added. This mixture was incubated for 10 minutes at ambient temperature before washing x 3 in PBS and resuspending in PBS.

The indirect haemolytic antibody titre was determined by the method described for the haemolytic antibody titre to SRBC except that 2% tanned SRBC were used. Preliminary tests showed that normal mouse serum did not lyse the PVP coated tanned SRBC and that a pool of serum from 10 mice immunized with PVP did not lyse the untreated tanned SRBC. The reaction required complement and could be completely inhibited by adding PVP (MW 24,500) at a final concentration of 2.5mg/ml.

H. Preparation of rabbit anti-SRBC antiserum fractions

Two rabbits were immunized intravenously with 10^9 SRBC and then intraperitoneally with 2×10^7 SRBC after 12 days. Serum was collected from the animals 12 and 27 days after the first inoculations. All sera were pooled and fractionated by Sephadex G-200 chromotography using PBS as an eluent. 3ml of serum was fractionated in a column (2.5cm x 100cm), by the upward flow method at a flow rate

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of 15ml/hour. 6ml fractions were collected and monitored for absorption at 280nm in a spectrophotometer. Three well defined peaks were obtained. The eluates were pooled into 2 fractions as shown (Fig. 3) and concentrated by ultrafiltration using a Diaflow PM-10 membrane (Diaflow Ultrafiltration membranes, Amicon, Lexington, Massachusetts, U.S.A.). Fraction I and fraction II (Fig. 3) were designated 19S and 7S fractions respectively as other studies have shown that these fractions would contain 19S or 7S antibody (Thomas, Turner, Eadie and Yadav 1972).

I. Phytohaemagglutinin (PHA) stimulation

The micromethod used to determine the incorporation of ^{3}H-6-thymidine (Radiochemical Centre, Amersham, U.K.) into lymphocytes in the presence of PHA-P (Difco Laboratories, Detroit, U.S.A.) has been described in detail by Keast and Bartholomaeus (1972). Mice were killed by cervical dislocation and 20IU of sodium heparin inoculated into the thorax and the heart severed. Blood was collected from the thoracic cavity. The spleens and lymph nodes of mice were finely minced using aseptic techniques, and suspended in 3ml of growth medium (RPMI 1640 (Grand Island Biological Co., New York, U.S.A.)) supplemented with 10% heat-treated calf serum (60°C for 2 hours). After allowing large tissue fragments to sediment by gravity the cell suspensions were purified by centrifugation on a mixture of Ficoll (Pharmacia, Uppsala, Sweden), and Hypaque (Winthrop Laboratories, New York, U.S.A) at 500g for 25 minutes (1ml of 34% (w/v) Hypaque to 2.4ml of 9% (w/v) Ficoll). The blood was made up to 3ml with growth medium and purified by centrifugation on a mixture of Ficoll and Hypaque. The leucocyte preparations were washed x 2 in growth medium (using centrifugation at 200g for 10 minutes) and resuspended in the medium. 5×10^{4} lymphocytes were then cultured in 0.2ml of medium containing either 0, 0.25, 1.25, 2.5, 4, 10, 20 or 50µg of PHA, in wells of Microtest II trays (Falcon Plastics Cat. No. 3040 and 3041). Triplicate cultures were set up for each concentration of PHA. After 24 hours incubation 0.5µCi of ^{3}H-6-thymidine (5Ci/mmole) was added to each culture and incubation continued for a further 24 hours. The plates were then centrifuged at 600g for 10 minutes using a centrifuge fitted with an attachment to hold Microtest plates (Cooke Engineering,

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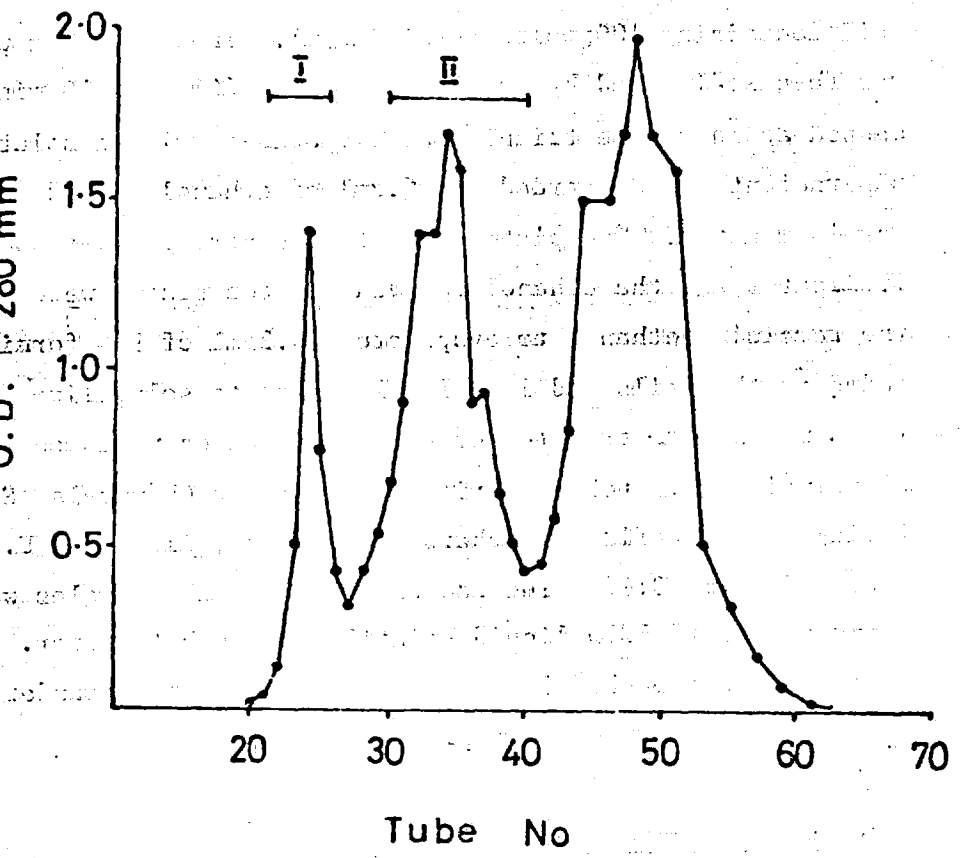

FIGURE 3 Fractionation of 3ml of rabbit serum in a Sephadex-G200 column (2.5 x 100cm). Tube volume 6.0ml.

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Alexandra, Virginia U.S.A.). The supernatant was removed and the cells washed x 2 in PBS containing 100μmoles thymidine/ml. 5μl of a 25mg/ml solution of bovine serum albumin were then added to the cell pellet in each well followed by 0.2ml of 5% trichloroacetic acid containing 100μmoles thymidine/ml. The resulting precipitate was then sedimented by centrifugation at 600g for 10 minutes and washed again in the trichloroacetic acid-thymidine solution. The supernatant was discarded and 0.2ml of ethanol was added to wells for 10 minutes. The plates were then centrifuged at 600g for 10 minutes and the ethanol removed and the plates were left for the remaining ethanol to evaporate. 0.25ml of 90% formic acid were added to the wells and left for 24 hours to solubilize the samples. The samples were transferred to scintillation vials containing 10ml of scintillant: toluene:triton-X 100 (Ajax Chemicals, Sydney, Australia) and Permafluor (Packard Instruments, Illinois, U.S.A.) in a ratio of 332:150:18. The radioactivity in the samples was determined using a Packard 3375 liquid scintillation spectrometer. Counting efficiency approached 50% and automatic external standardisation was carried out on each sample.

J. Pulmonary-bacteriocidal activity

A leucine-requiring strain of *Pseudomonas aeruginosa* was cultured overnight in a nutrient broth containing 50μCi/ml of H^3-5-uridine (Radiochemical Centre Amersham, Bucks U.K., 5Ci/mmole), then washed three times in PBS and resuspended in PBS at a concentration of $2x10^9$ bacteria/ml. The number of bacteria was measured with a Helber chamber. Mice were anaesthetised with pentobarbitone sodium and a blunt 19 gauge needle inserted under the epiglottis and almost to the base of the trachea, where 0.01ml of the bacterial suspension was introduced. After a specified time interval, the lungs were then removed from just above the junction of the trachea and bronchi, and homogenised in 0.5ml of PBS. To measure radioactivity, 0.05ml of the homogenate was incubated at 37°C with 0.05ml of hydrogen peroxide for 2 hours, then 1.0ml of soluene (Packard Instruments, Illinois, U.S.A.) was added and the mixture incubated for a further 22 hours. The sample was then transferred to a scintillation vial containing 10ml of a 1:24 mixture

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of Permafluor (Packard Instruments) and toluene. Radioactivity was determined in a Packard 3375 liquid scintillation spectrometer. External standardisation was carried out on each sample and counting efficiency approached 30%. The viable-bacterial counts were performed by serially diluting the lung homogenates in PBS and plating 0.1ml of an appropriate dilution on a minimal media plate (containing 1.5% agar in a Vogel and Bonner salt solution (Vogel and Bonner 1956)) with 0.5% glucose, 0.01% leucine and 0.03% cetrimide (cetrimonium bromide) and counting the colonies growing after an overnight incubation. When the dilutions were plated on a plate without leucine, the number of colonies was negligible, indicating that almost all the bacteria normally counted were the leucine-requiring strain originally introduced to the lungs. The counts of viable bacteria and radioactivity were performed in duplicate, and the values compared to a group processed immediately after the administration of bacteria.

K. Systemic reticuloendothelial function

2×10^8 bacteria from a preparation of *Pseudomonas aeruginosa* which had been labelled by incubation with H^3-5-uridine, were inoculated into the tail vein of mice and immediately (t_0) 20µl of blood was sampled from the retro-orbital plexus. After 10 minutes (t_{10}) another sample was taken. The phagocytic index was calculated by measuring the disappearance of radioactivity from the blood.

$$K_p = \frac{\log DPM_0 - \log DPM_{10}}{t_{10} - t_0}$$

(t = minutes, DPM = disintegrations per minute).

At 10 minutes and 180 minutes after the inoculation, the radioactivity in the spleen, kidney, liver and lungs was determined. The radioactivity in the organs as well as the blood was counted as described above for the lungs (Section I). The change of the radioactivity in the organs between 10 and 180 minutes was analysed by calculating the slope and standard deviation of the slope of the regression line of radioactivity on time. The significance of the differences in the slopes between control and smoke-exposed animals was calculated by Student's t test (Campbell 1967).

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L. *In vitro* phagocytosis and elimination of radioactivity

The method used to measure phagocytosis *in vitro* has been described in detail (Thomas, Holt and Keast 1974b). Peritoneal macrophages were obtained from untreated C57 Black mice and cultured in MEM supplemented with 10% heat treated calf serum (2 hours, 60°C), and cultured on stainless steel squares in 35mm petri dishes containing 2ml of medium as described by Keast and Birnie, (1969) for 20 - 22 hours. Batches of 5 - 15 mice were used for each experiment. Macrophages were plated in petri dishes without stainless steel squares and by counting the number of viable macrophages, as judged by trypan blue exclusion, in 20 fields of 3 dishes, the number of macrophages per stainless steel square was determined (Keast and Birnie 1969). To measure phagocytosis, heat-treated *Pseudomonas aeruginosa* were added to the cultures after changing the medium. Except where indicated 18.8×10^7 bacteria/ml were used. After the required period for phagocytosis, extracellular bacteria were washed off by directing a stream of PBS (1ml) twelve times across the stainless steel square. The cultures were further rinsed in PBS, fixed in 5% trichloroacetic acid for 10 minutes and then in 3:1 (v/v) ethanol/acetone for 3 minutes before rinsing in acetone. To measure elimination of phagocytosed radioactivity, the macrophages were washed as above and then reincubated in fresh warm medium. After the period of elimination the macrophages were rinsed in PBS and then fixed and dehydrated as above. The radioactivity was measured with a Nuclear Chicago gas-flow detector (Model D47). The specific activity of the bacterial preparations were determined and the results expressed as bacterial equivalents/cell. No cell death occurred when the macrophages were incubated with the bacteria for 5 hours but 60% macrophage death occurred if the incubation with bacteria was continued for 24 hours. However, when macrophages were incubated with the bacteria for 3 hours, then washed to remove extracellular bacteria and reincubated for 24 hours in fresh medium no cell death occurred.

In these studies radiolabelled *Pseudomonas aeruginosa* were prepared by incubating the bacteria in a minimal salts solution (Vogel and Bonner 1956) with 0.5% glucose, 0.001% leucine and 20μCi

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of H^3-5-uridine. The bacteria were heat-treated for 2 hours at 60°C, washed three times in PBS and stored at 4°C.

To study the effect of exposure to cigarette smoke on the phagocytic capacity of macrophages, their phagocytosis was measured 30 minutes after the exposure unless otherwise specified. The adherence of the macrophages was measured by counting the number of viable macrophages before and after the washing process used to remove bacteria. The adherence was calculated and expressed as the percentage of macrophages remaining on the monolayers after the washing.

M. Growth of tumours

The Lewis lung tumour (Sugiura and Stock 1955) of C57Black mice was a gift from Dr. Hellman of the Imperial Cancer Research Fund, Lincolns Inn Fields, London, U.K., and was maintained by serial transplantation in adult mice. This tumour originated as a spontaneous anaplastic lung carcinoma. A preliminary study showed that the subcutaneous inoculation of 10^6 viable cells produced tumours in 18/20 mice. This number of viable cells was used as a standard inoculum. The diameter of the tumours was determined by two measurements taken at right angles to each other, employing calipers. The cube of the mean of the diameters determined by this method and the weight of the tumours were found to have a product-moment correlation coefficient of 0.89. The ability of this tumour to metastasise to the lungs was measured by the method of Wexler (1966). The lungs were infused with dilute India ink (15ml of ink in 85ml of distilled water) through the trachea, washed in tap water and then fixed and bleached in Feketes solution (70% ethanol, formaldehyde, acetic acid mixed in the ratio of 100:10:5). In order to allow the tumours maximum opportunity to metastasise, the tumour bearing animals were left until a number died or became moribund (about 30 days).

The TKL5 cells were a clone from a line (W47-A) of tumour cells cultivated *in vitro* from a tumour induced in a newborn BALB/c mouse by murine sarcoma virus (Harvey) (MSV-H) (Thomas, Aw, Papadimitriou and Simons 1973). They were maintained in tissue culture using

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McCoys 5A medium (Grand Island Biological Company, New York, U.S.A.), supplemented with 10% heat-treated calf serum (2 hours, 60°C), and produced MSV-H as judged by the ability of tissue-culture-fluid filtrates to produce tumours in newborn mice. These cells were inoculated intratracheally (Section F(a)). The standard inoculum was 10^5 viable cells per mouse.

N. Histopathology

Histological examination was performed by the techniques of Walters, Leak, Joske and Stanley (1963).

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CHAPTER 3

EXPERIMENTAL MODEL FOR CIGARETTE-SMOKE -INHALATION STUDIES

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A. Introduction

Attempts to approximate the smoking habit of humans have involved forcing or training animals to inhale air through a burning cigarette (Jarvick 1967; Albert *et al* 1969; Auerbach *et al* 1970; Stuart, Willard and Howard 1970). This has been accomplished with the aid of masks and tracheotomies (Albert *et al* 1969; Auerbach *et al* 1970a; Stuart *et al* 1970). These active inhalation procedures are suitable for large animals such as dogs (Auerbach *et al* 1970a) and donkeys (Albert *et al* 1969) but are time consuming, expensive and unsuitable for small laboratory animals (Hoffmann and Wynder 1970).

Several passive inhalation techniques have been developed to force small animals to inhale cigarette smoke generated in an exposure chamber (Hoffmann and Wynder 1970). Since particulate aggregation as well as physical and chemical aging occurs in cigarette smoke it was necessary to develop machines which could present animals with a fresh cigarette smoke (Hoffmann and Wynder 1970). A number of machines designed to produce fresh mixtures of cigarette smoke and air have been developed and used to produce pathological changes in small rodents (Harris and Negroni 1967; Dontenwill 1970; Leuchtenberger and Leuchtenberger 1970a; Dontenwill *et al* 1973).

In the experiments described in this thesis, a Hamburg II smoking apparatus (Dontenwill 1970), which was commercially available (Heinr Borgwaldt, West Germany), was used to expose mice to cigarette smoke. The machine was set to present puffs of fresh cigarette smoke in a 1:7 smoke:air ratio, with a constant rhythm of smoke flow. On five consecutive days of each week mice were exposed to the puffs from 30 cigarettes (7 minutes continuous smoke exposure per day). The smoke exposure conditions were intended to subject the mice to acceptable concentrations of cigarette smoke while avoiding acute toxic effects and permitting the mice to live an acceptable life span (Dontenwill 1970).

It was, however, considered necessary not only to standardize the exposure conditions but also to submit the mice to a battery of tests which could assist in determining the amount of smoke inhalation and the sensitivity of the animals to the toxic effects of cigarette smoke. Therefore the measurements of blood carboxyhaemoglobin, body weights, organ weights, food consumption

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and haematological values of mice exposed to cigarette smoke which are described in this chapter were determined to help define the murine model used for the immunological studies.

B. Blood carboxyhaemoglobin

The carboxyhaemoglobin concentration (%HbCO) in the peripheral blood of BALB/c mice was measured after 1/2, 1, 2 and 3 rounds of exposure to cigarette smoke (Fig. 4) (1 round of cigarettes is the exposure of the mice to 30 cigarettes over a standardised time of 7 minutes). The carboxyhaemoglobin concentrations were also measured for up to an hour after the third round of cigarettes (Fig. 4). The mice developed high levels of carboxyhaemoglobin (30.6% of the haemoglobin) but these rapidly declined after discontinuing the exposure to cigarette smoke. The concentration of carboxyhaemoglobin was measured in 6 strains of mice after 1 round of cigarette smoke exposure (Table 1). The carboxyhaemoglobin levels of C57Black C3H and DK Black mice were significantly higher than the levels in BALB/c mice ($p<0.05$). The C57Black mice also had a significantly higher concentration than Simpson mice ($p<0.05$).

C. Toxicity of cigarette smoking

To measure the acute toxic effects of the inhalation of cigarette smoke, C57Black mice were continuously exposed to cigarette smoke. Generally 8 rounds of cigarette-smoke exposure was lethal for 50% of the mice (56 minutes of continuous exposure to cigarette smoke). No controlled toxicity study for chronic exposure was performed but few mice died after periods of exposure up to 42 weeks. Generally all the mice appeared to be in good condition after chronic exposure to cigarette smoke.

D. Body-weight and diet

The body weight and diet of 8 week old C57Black mice were measured after periods up to 80 days of exposure to cigarette smoke (Table 2). The mice exposed to cigarette smoke lost some weight and maintained a lower body-weight than age matched controls. Mice

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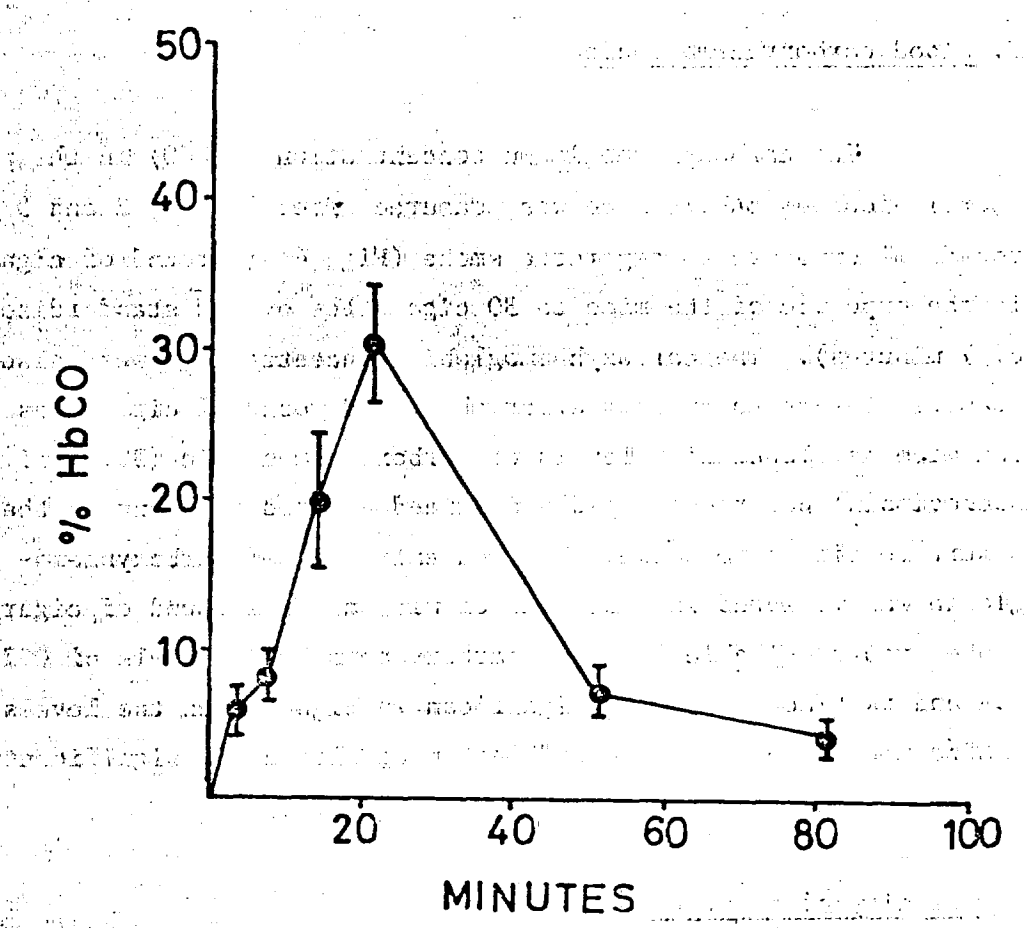

FIGURE 4 Blood carboxyhaemoglobin levels in BALB/c mice exposed to cigarette smoke. Mice were exposed for 1/2 (3.5 minutes), 1 (7 minutes), 2 (14 minutes) and 3 rounds of cigarettes (21 minutes), in a Hamburg II smoking machine. Each result is the mean ± S.E. of the values from 5 mice: † smoke-exposure discontinued.

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TABLE 1 Blood carboxyhaemoglobin in six strains of mice after inhalation of cigarette smoke

Mouse strain	% Carboxyhaemoglobin (mean ± S.E.)
1. BALB/c	8.7 ± 2.1
2. Simpson	12.5 ± 3.3
3. DK Ginger	17.4 ± 2.4
4. DK Black	20.0 ± 3.1
5. C3H	21.7 ± 2.1
6. C57Black	22.7 ± 2.8

The significance of differences between the % carboxyhaemoblobin was examined by Student's t test. Strains 4 and 5 were significantly different from strain 1 ($p<0.05$). Strain 6 was significantly different from strain 1 and 2 ($p<0.05$).

TABLE 2 Body weight and diet of C57Black mice exposed to cigarette smoke

Days smoke exposure	Group	Body-weight (g)	Food consumption (g/mouse/day) (moist wt.)(2)
0	exposed	18.4 ± 0.3(1)	
	control	18.3 ± 0.06	
10	exposed	17.7 ± 0.3***	2.0 ± 0.01**
	control	19.4 ± 0.2	2.7 ± 0.1
24	exposed	17.9 ± 0.2***	2.0 ± 0.03*
	control	19.7 ± 0.2	2.4 ± 0.03
44	exposed	18.5 ± 0.3***	
	control	20.5 ± 0.2	
80	exposed	18.6 ± 0.3***	1.9 ± 0
	control	21.4 ± 0.3	2.0 ± 0.1

(1) Each result is the mean ± S.E. of the values from 20 mice.
(2) The food consumption of cages of mice was measured over a period of one week. Each result is the mean ± S.E. of the values from 3 cages.

The significance of the differences between the values from control mice and mice exposed to cigarette smoke was determined by Student's t test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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exposed to cigarette smoke also had a 7.6% lower body weight than the controls when measured after 28 weeks cigarette-smoke exposure. The weight of mouse food-cubes consumed by groups of mice exposed to cigarette smoke and control mice during one week was measured after 10, 24 and 80 days of exposure to cigarette smoke (Table 2). The mice exposed to cigarette smoke had a decreased food intake.

E. Organ weights

The weights of the lungs, spleens, livers and kidneys of C57Black female mice exposed to cigarette smoke for 18 and 34 weeks were measured (Tables 3,4). No significant differences were found between the weights of organs from cigarette-smoke exposed or age-matched-control mice.

F. Haematology

The haematological data of groups of 10 - 20 mice were determined after 8, 20, 24 and 35 weeks cigarette-smoke exposure (Tables 5, 6, 7, 8). The changes occurring in leucocyte and erythrocyte counts, haemoglobin concentration, packed-cell volumes and mean cell volumes are summarised in Table 9. The erythrocyte counts and concentrations of haemoglobin of mice exposed to cigarette smoke showed a transient increase but after 35 weeks exposure fell to below the levels of age-matched-controls. The leucocyte count was depressed when measured after only 8 weeks inhalation of cigarette smoke. No differences in the differential leucocyte counts of control mice and mice exposed to cigarette smoke were found.

G. Discussion

Experimentation into the effect of cigarette smoke on laboratory animals has depended on the development of machines which can force animals to inhale a suitable amount of fresh cigarette-smoke (Leuchtenberger and Leuchtenberger 1968; Dontenwill 1970; Hoffmann and Wynder 1970). The Hamburg II inhalation apparatus (Dontenwill 1970) used in the experiments of this programme has been described as one of the most advanced designs of inhalation chambers

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TABLE 3 Organ weights in C57Black mice exposed to cigarette smoke for 18 weeks

Organ	Organ weight (g moist weight)* Smoke-exposed	Control
Lungs	0.19 ± 0.01	0.17 ± 0.02
Liver	0.89 ± 0.03	0.93 ± 0.05
Kidney	0.25 ± 0.02	0.24 ± 0.02
Spleen	0.102 ± 0.01	0.094 ± 0.02

* Each result is the mean ± S.E. of values from 6 mice. No significant difference was found between the organs of test and control mice as judged by Student's t test.

TABLE 4 Organ weights in C57Black mice exposed to cigarette smoke for 34 weeks

Organ	Organ weight (g moist weight)* Smoke-exposed	Control
Lungs	0.17 ± 0.02	0.19 ± 0.01
Liver	0.93 ± 0.08	1.07 ± 0.04
Kidney	0.25 ± 0.02	0.25 ± 0.02
Spleen	0.087 ± 0.02	0.109 ± 0.02

* Each result is the mean ± S.E. of values from 6 mice. No significant difference was found between the organs of cigarette smoke exposed and control mice as judged by Student's t test.

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TABLE 5 Haematological data of C57Black female mice exposed to cigarette smoke for 8 weeks

Specification		Exposed	Control
Erythrocyte count (N^o/mm^3)x10^{-6}		9.8 ± 0.07 (1)	9.7 ± 0.07
Haemoglobin concentration (g/100ml)		15.0 ± 0.11	14.9 ± 0.09
Packed cell volume (%)		44.2 ± 0.33	45.0 ± 0.29
Mean cell volume (μ^3)		44.9 ± 0.04	45.2 ± 0.02
Mean cell haemoglobin (µµg)		15.6 ± 0.04	15.6 ± 0.03
Mean cell haemoglobin concentration (%)		33.1 ± 0.09	32.9 ± 0.09
Leucocyte count (N^o/mm^3)x10^{-3}	***	7.8 ± 0.37	10.6 ± 0.45
Differential leucocyte count			
Neutrophils (%)		10.7 ± 0.90	11.9 ± 1.1
Lymphocytes (%)		85.4 ± 1.1	84.6 ± 0.90
Monocytes (%)		4.1 ± 0.45	3.7 ± 0.56

(1) Each result is the mean ± S.E. of the values from 10 mice. The significance of the difference between smoke-exposed and control animals was examined with Student's t test: * $p<0.05$, *** $p<0.001$.

TABLE 6 Haematological data of C57Black female mice exposed to cigarette smoke for 20 weeks

Specification		Exposed	Control
Erythrocyte count (N^o/mm^3x10^{-6})		9.9 ± 0.09 (1)	9.8 ± 0.09
Haemoglobin concentration (g/100ml)		14.4 ± 0.13	14.2 ± 0.16
Packed cell volume (%)	*	45.9 ± 0.36	44.3 ± 0.40
Mean cell volume (μ^3)	*	45.4 ± 0.25	44.5 ± 0.11
Mean Cell haemoglobin (µµg)		14.8 ± 0.16	14.6 ± 0.07
Mean cell haemoglobin concentration (%)		31.2 ± 0.16	31.9 ± 0.13
Leucocyte count (N^o/mm^3x10^{-3})	*	4.8 ± 0.31	6.7 ± 0.36
Differential leucocyte count			
Neutrophils (%)		13.5 ± 1.6	12.6 ± 1.1
Lymphocytes (%)		85.1 ± 1.5	85.9 ± 1.2
Monocytes (%)		1.4 ± 0.31	1.5 ± 0.25

(1) Each result is the mean ± S.E. of the values from 20 mice. The significance of the difference between the smoke-exposed and control groups was tested by Student's t test: * $p<0.05$.

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TABLE 7 Haematological data of C57Black male mice exposed to cigarette smoke for 23 weeks

Specification		Exposed	Control
Erythrocyte count ($N^o mm^3 x10^{-6}$)	***	9.7 ± 0.09	9.3 ± 0.07
Haemoglobin concentration (g/100ml)	***	14.3 ± 0.09	13.5 ± 0.07
Packed cell volume (%)	***	45.1 ± 0.38	42.5 ± 0.25
Mean cell volume (μ^3)	**	44.4 ± 0.11	43.9 ± 0.09
Mean cell haemoglobin (µµg)		14.8 ± 0.03	14.7 ± 0.04
Mean cell haemoglobin concentration (%)		31.4 ± 0.09	31.4 ± 0.09
Leucocyte count ($N^o/mm^3 x10^{-3}$)	**	7.1 ± 0.38	8.9 ± 0.40
Differential leucocyte count			
Neutrophils (%)		12.4 ± 1.1	14.6 ± 1.1
Lymphocytes (%)		84.9 ± 1.0	82.1 ± 1.2
Monocytes (%)		2.8 ± 0.34	3.2 ± 0.42

Each result is the mean ± S.E. of the values of 20 mice. The significance of the difference between the smoke-exposed group and the controls was examined by Student's t test: ** $p<0.01$, *** $p<0.001$.

TABLE 8 Haematological data of C57Black male mice exposed to cigarette smoke for 35 weeks

Specification		Exposed	Control
Erythrocyte count ($N^o/mm^3 x10^{-6}$)	**	9.5 ± 0.11	10.1 ± 0.19
Haemoglobin concentration (g/100ml)	*	14.1 ± 0.16	14.8 ± 0.28
Packed cell volume (%)	*	43.8 ± 0.56	46.2 ± 0.85
Mean cell volume (μ^3)		44.6 ± 0.20	44.6 ± 0.16
Mean cell haemoglobin (µµg)	**	14.9 ± 0.07	14.7 ± 0.02
Mean cell haemoglobin concentration (%)		32.2 ± 0.16	32.0 ± 0.09
Leucocyte count ($N^o/mm^3 x10^{-3}$)	***	6.4 ± 0.36	9.0 ± 0.52
Differential leucocyte count			
Neutrophils (%)		15.2 ± 1.2	13.0 ± 0.94
Lymphocytes (%)		81.6 ± 1.6	84.4 ± 1.2
Monocytes (%)		3.2 ± 0.47	2.6 ± 0.45

Each result is the mean ± S.E. of the values from 20 mice. The significance of the difference between the smoke-exposed and control mice was examined by Student's t test:
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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TABLE 9 Haematological data of C57Black mice exposed to cigarette smoke expressed as a percent of the values of control mice

	Weeks exposure to smoke			
	8 week	20 week	23 week	35 week
Leucocyte count	*** 73.6	* 71.6	** 80.0	*** 71.1
Erythrocyte count	101.0	101.0	*** 104.0	** 94.0
Haemoglobin concentration	100.7	* 101.4	*** 106.0	* 95.0
Packed cell volume	98.2	* 103.6	** 106.1	* 94.8
Mean cell volume	99.0	* 102.0	101.0	100.0

The values shown are derived from data presented in Tables 5 - 8. Each value is the mean of the values from a group of mice exposed to cigarette smoke shown as a percentage of the corresponding mean control value. The significance of the differences between the smoked mice and controls was determined by Student's t test.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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(Hoffmann and Wynder 1970). There are, however, obvious reasons for expecting that the experimental model used does not duplicate the human cigarette-smoking habit. Passively inhaling the smoke produced from 30 cigarettes for 7 consecutive minutes each day is different from the human habit, where cigarettes are typically smoked intermittantly during the day. Because the diameters and lengths of the airways, as well as the respiration rates of mice, are different from humans the distribution and depositions of the components of cigarette smoke in humans and mice are probably different (Dontenwill 1970). In any case the differences which distinguish mice from men also probably ensure that they react differently with the components of cigarette smoke.

Experimental and epidemiological studies of the effect of cigarette-smoke inhalation on humans are limited by factors such as genetic and cultural variations of humans, their different smoking habits and the inconvenience and discomfort produced by some experimental procedures. In addition to the availability of inbred animals, the use of experimental animals can also present the opportunity to study phenomena which are isolated or pronounced in animals but not in humans. Animals can be chosen for an experimental model because their size, availability and longevity may assist experimentation. As cigarette-smoke probably has different effects on different animals the use of several different animal models may be required to investigate the various aspects of cigarette smoking. This argument also applies to the different methods used to expose animals to cigarette smoke. Variation in the effect of cigarette smoke on different animals is illustrated by the large differences in the carboxyhaemoglobin levels produced in different strains of mice by cigarette-smoke inhalation (Table 1). Similar results have been reported (Dontenwill 1970). A standard regime of cigarette-smoke exposure has also been found to produce increased incidence of neoplasia in one strain of mice and cardiovascular disease in another (Leuchtenberger and Leuchtenberger 1974). While these results emphasise the difficulty in obtaining a comprehensive experimental-animal model for studying the effect of cigarette-smoke inhalation, they also indicate that the effect of cigarette smoking on different humans may be quite varied. This may be important in determining types of people who are at risk of developing the

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diseases associated with cigarette smoking.

The level of over 20% carboxyhaemoglobin which was reached by C57Black mice after one exposure to cigarette smoke shows that the mice were receiving at least an adequate exposure to the vapour phase of cigarette smoke. This level is not often achieved by humans and can produce neurological symptoms (Peterson and Stewart 1970; World Health Organization 1972). The study on carboxyhaemoglobin as well as body-weight, diet and toxicity produced results very similar to those of Dontenwill (1970), Reckzeh and Dontenwill (1970) and Dontenwill (1973). The mice exposed to cigarette smoke had depressed body-weights, associated with a decreased intake of food, but no differences were found between weights of organs from control mice and from mice exposed to cigarette smoke. However the errors associated with the estimation of the mean body-weights were less than the errors of estimating the mean organ-weights. Exposure to cigarette smoke also produced an increase in the erythrocyte counts and haemoblobin concentrations of mice, similar to the increase of erythrocyte count found in hamsters exposed to cigarette smoke (Reckzeh and Dontenwill 1970; Dontenwill *et al* 1973). However, prolonged exposure to cigarette smoke (35 weeks) decreased the erythrocyte counts and haemoglobin concentrations of the mice. Hamsters, exposed to cigarette smoke for a comparable period did show reductions in erythrocyte and haemoglobin levels (Dontenwill *et al* 1973). Although mice exposed to cigarette smoke eventually had a lower erythrocyte counts and haemoglobin concentrations than age-matched-control mice the values were well within the normal range. The most marked change in the haematology of the mice exposed to cigarette smoke was the large decrease in the number of peripheral-blood leucocytes. Reckzeh and Dontenwill (1970) and Dontenwill *et al* (1973) reported that leucocyte counts of hamsters were not significantly decreased by cigarette-smoke inhalation. Their results showed that the animals exposed to cigarette smoke consistantly had lower leucocyte counts than the controls and the counts became close to control levels after discontinuing the exposure to cigarette smoke (Reckzeh and Dontenwill 1970). In contrast to mice, cigarette smokers have an elevated number of peripheral-blood leucocytes (Corre *et al* 1971; Friedman *et al* 1973) but there is evidence indicating that this may be caused by the increased incidence

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of respiratory infection in cigarette smokers (Friedman *et al* 1973).

Although the carboxyhaemoglobin of mice exposed to cigarette smoke was high, the daily 7 minute exposures to cigarette smoke were considerably below acute lethal doses. This daily exposure could produce demonstrable effects on the mice, such as changes in body weight, but the mice were apparently healthy and could be maintained in experiments for prolonged periods.

due to CO

Taking into account the similarities between these observations and those of other studies, as well as the large body of evidence implicating cigarette smoking as the cause of a wide variety of diseases (Royal College of Physicians 1971; United States Public Health Service 1971), it was concluded that the experimental system was suitable for studying the effect of inhalation of cigarette smoke on the immune system of mice.

Question

rationale for using mouse inhal. system only, hence

each model was evaluated against the similarity of effects in mice

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CHAPTER 4

IMMUNOLOGICAL STUDIES

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SECTION 1 HUMORAL IMMUNITY

A. Introduction

The initial experiments described in this section are concerned with immune responses of normal mice. In order to study the effects of cigarette smoke on the immune responses of mice, it was considered important to examine the local and systemic immune responses to immunogens introduced into the respiratory system. Accordingly, the ability of intratracheal inoculations of sheep erythrocytes (SRBC) to elicit an antibody-forming-cell response in the lungs, the lymph nodes draining the respiratory system, the spleen and blood of mice was determined. The Cunningham and Szensberg modification of the Jerne plaque technique (Cunningham and Szensberg 1968) was employed to study the antibody-forming-cell responses. Both direct and indirect plaque-forming cells (pfc) were examined which can generally be equated with IgM antibody-producing cells and IgG or IgA antibody-producing cells respectively (Sterzl and Riha 1965). The nature of the responses was examined further by determining the effect of combinations of intravenous and intratracheal inoculations.

The humoral immune responses, of mice chronically exposed to cigarette smoke, to intratracheal inoculations of SRBC as well as to intraperitoneal inoculations of SRBC were then studied. As well as the responses to SRBC, an immunogen considered to require thymus-dependent lymphocytes (T-cells) (Davies, Leuchars Wallis and Doenhoff 1971; Playfair 1971), the antibody response to a T-cell independent immunogen, polyvinylpyrrolidone (PVP) (Andersson and Blomgren 1971), was also studied.

B. Local and systemic immune response of mice after intratracheal and intravenous inoculations of SRBC

In this section the antibody-forming-cell response, as estimated by the numbers of haemolytic-plaque-forming cells (pfc), occurring in normal mice after intratracheal and intravenous inoculations of sheep erythrocytes is described. The pfc in cell suspensions prepared from the lungs, spleen, blood and mediastinal and cervical lymph nodes were examined. The reproducibility of the

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intratracheal inoculation can be judged by the results of experiments described in Chapter 4 section 3.B where the technique has been used to deliver radioactive bacteria to the lungs.

(a) Plaque-forming cells in non-immunized mice

Plaque-forming cells were not detected in the lungs, blood or pools of mediastinal and cervical lymph nodes of 5 normal C57Black mice. In the spleens 1.6 ± 0.2 direct pfc/10^6 (mean ± S.E.) leucocytes were detected.

(b) Lymph nodes responding to intratracheal inoculations

Groups of five C57Black mice were inoculated intratracheally with 10^8 SRBC then examined 7 or 9 days later. The mediastinal, cervical and axillary lymph nodes were removed and cell suspensions prepared separately and suspended in a final volume of 0.1ml of growth medium. They were then tested for direct and indirect pfc (Table 10). The largest and most consistent response was found in the mediastinal lymph nodes when considered on a per organ or per 10^6 leucocytes basis. The cervical lymph nodes also made a large contribution to the response. However axillary lymph nodes had little or no response to intratracheal inoculations.

(c) Response in the spleen, lungs, blood and cervical and mediastinal lymph nodes to intravenous and intratracheal inoculations of SRBC

C57Black mice were inoculated intravenously or intratracheally with SRBC and then on day 12, mice from each group were challenged either intravenously or intratracheally. Groups of five mice from the four inoculation regimes were removed on days 3, 6, 7, 12, 15, 18, 21 and 24 after the first inoculation. They were examined for direct and indirect pfc in the lungs, blood, spleen and the pool of cervical and mediastinal lymph nodes. In order to test the correlation of pfc in organs and pfc freely circulating in the blood the results of direct and indirect pfc were regarded as separate values in the same set and the product-moment correlation coefficient (r) was calculated (Campbell 1967). Each value in the results (Figs. 5 - 8) represents the mean ± S.E. of values from 5 mice.

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TABLE 10 Plaque-forming cells in lymph nodes after an intratracheal inoculation of SRBC

	Days after inoculation	Lymph Nodes					
		Mediastinal		Cervical		Axillary	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
pfc/organ	7	32 ± 22 (1)	31 ± 22	23 ± 19	7 ± 2	0	0
	9	20 ± 14	35 ± 33	2 ± 1	14 ± 6	0.5 ± 0.5	0
pfc/10^6 leucocytes	7	89 ± 53	82 ± 58	12 ± 7	4 ± 1	0	0
	9	43 ± 15	57 ± 42	2.4± 1	9.6± 2	1.9 ± 1.5	0

(1) Each value is the mean ± S.E. of the values from five mice.

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(i) Response of mice to intravenous inoculations (Fig. 5)

After the intravenous inoculation of 10^8 SRBC a large response occurred in the spleen with primarily direct pfc detected on day 3 followed by a predominantly indirect pfc response reaching a maximum on day 6. A small number of direct and indirect pfc was found in the blood on day 6 but few pfc were found in either the lymph nodes or the lungs. However, after a second intravenous inoculation, significant numbers of pfc were found in all organs examined. An anamnestic response occurred in the spleen for both direct and indirect pfc showing a maximum reaction after 3 days. The blood and the lungs showed the greatest number of pfc 6 days after the second inoculation. The blood had more pfc/10^6 leucocytes than the lungs, both having approximately equal proportions of direct and indirect pfc. The response shown in the lungs and blood was highly correlated ($r = 0.76$, $p<0.001$) (Campbell 1967). A response consisting primarily of indirect pfc was found in the lymph nodes, also being maximal at 6 days after boosting. However no relationship between the pfc found in the blood and lymph nodes was obvious ($r = 0.086$).

(ii) Response of mice to intratracheal inoculations (Fig. 6)

After intratracheal inoculation the spleen was still the organ containing the largest total number of pfc. However when considered per 10^6 leucocytes, the lymph nodes and the lungs had the most intense response. These organs had a transient response of both direct and indirect pfc being maximal on day 6. A small response of direct pfc was found in the blood on day 6.

Following a second intratracheal inoculation, an anamnestic response was found in the lungs and the lymph nodes showing an increased and prolonged indirect pfc involvement. No pfc appeared in the blood. The spleen showed a secondary response consisting primarily of indirect pfc, and still contained the largest number of pfc.

(iii) Intravenous inoculation of mice sensitized by intratracheal inoculation (Fig. 7)

The largest number of pfc found in the lungs and lymph nodes, after the inoculation regimes tested, was obtained when the animals were first inoculated intratracheally and then challenged intravenously. These responses consisted predominantly of indirect pfc.

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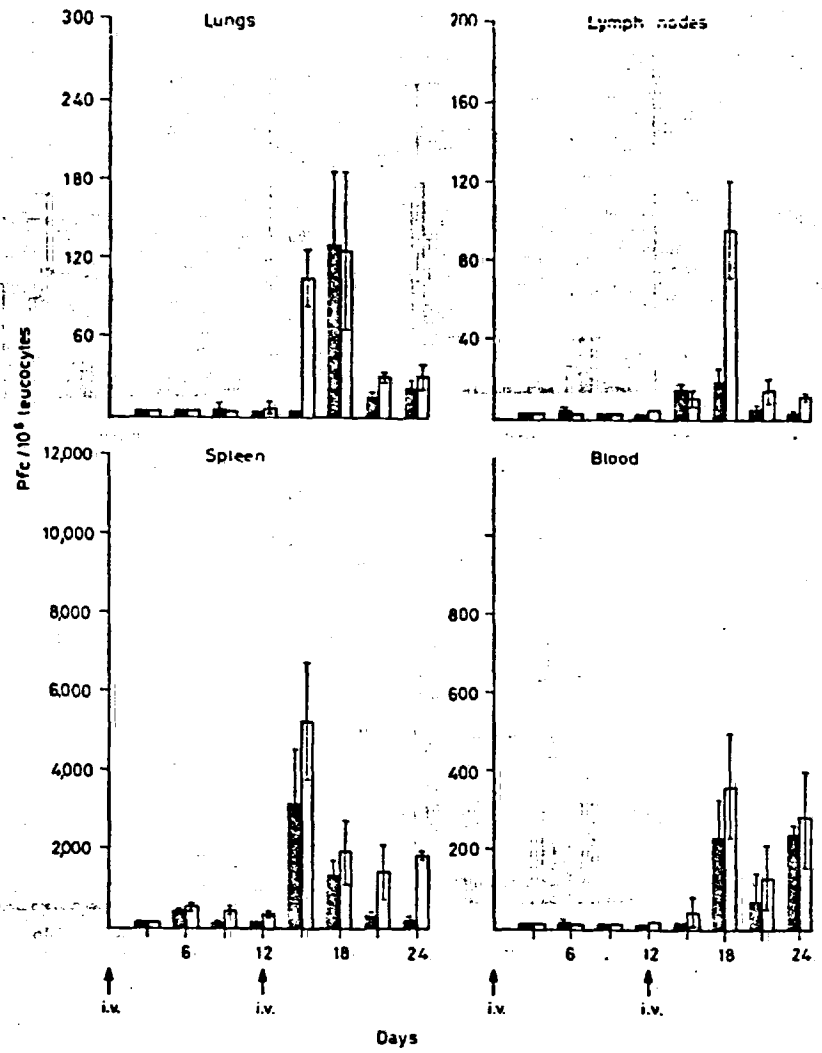

FIGURE 5 Plaque-forming-cell response in mice immunized with two intravenous (IV) inoculations of 10^8 SRBC; direct pfc ■ indirect pfc □ , ↑ indicates time of inoculation.

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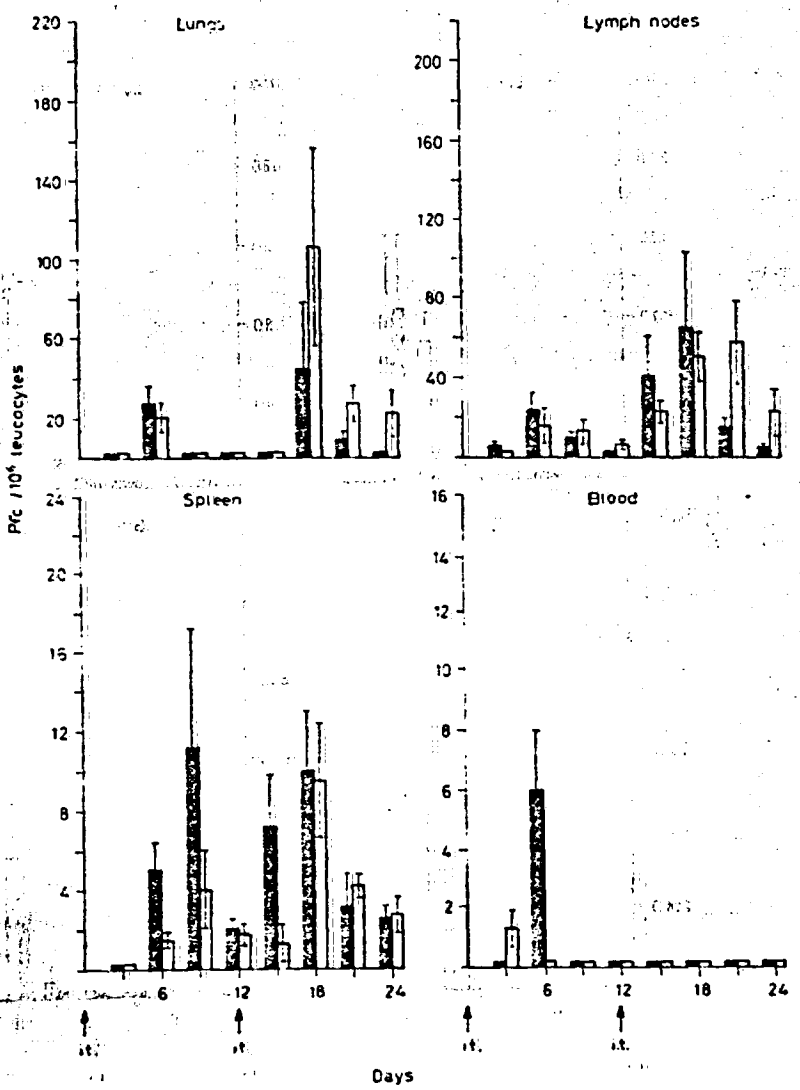

FIGURE 6 Plaque-forming-cell response in mice immunized with two intratracheal (IT) inoculations of 10^8 SRBC; direct pfc ■ indirect pfc □ , † indicates time of inoculation.

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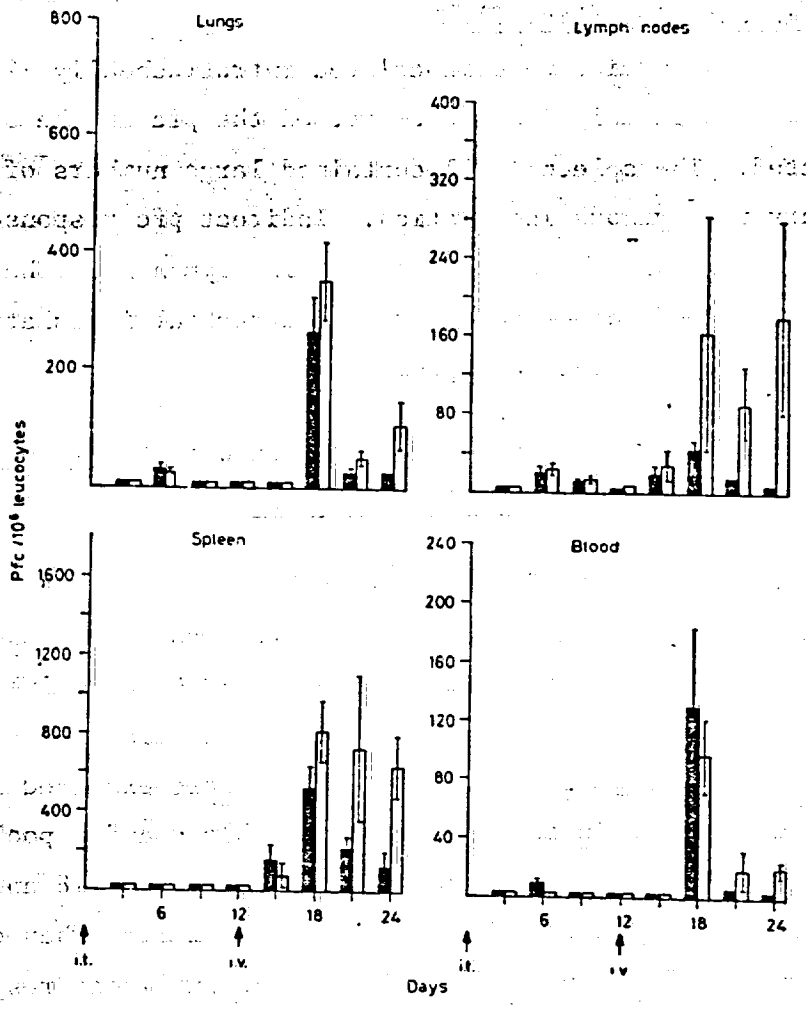

FIGURE 7 Plaque-forming-cell response in mice immunized with an intratracheal (IT) then an intravenous (IV) inoculation of 10^8 SRBC; direct pfc ■ , indirect pfc □ , † indicates time of inoculation.

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Although pfc persisted in the circulation after these inoculations, this response was not significantly correlated ($r = 0.46$) with the lung reaction. The lungs also contained more pfc/10^6 leucocytes than the blood. Sufficient priming of the spleen occurred after intratracheal challenge to produce an intense reaction after intravenous challenge.

(iv) Intratracheal inoculation of mice sensitized by intravenous inoculation (Fig. 8)

When mice were inoculated intratracheally 12 days after an intravenous inoculation, no effect on the pfc in the spleen could be detected. The spleen still contained large numbers of pfc from the primary intravenous inoculation. Indirect pfc responses were detected in the blood and the lungs. The lymph nodes had a response no greater than after a primary intratracheal inoculation, consisting predominantly of indirect pfc.

C. Humoral immune responses of mice exposed to cigarette smoke to intratracheal inoculations of SRBC

(a) Primary and secondary pfc response

C57Black mice were exposed to cigarette smoke for 26 weeks and inoculated intratracheally with 10^8 SRBC in 0.01ml of PBS and the smoking regime continued. A second inoculation was given, where necessary, on day 12. Groups of 5 mice were examined for direct and indirect pfc in the lungs, spleen, blood and a pool of the mediastinal and cervical lymph nodes at 7, 9, 12, 15, 18 and 21 days after the first inoculation. The results were similar when considered per organ or per 10^6 leucocytes. The only significant response found in the blood was in age-matched control mice 7 days after the inoculation (5 ± 2 direct pfc/10^6 leucocytes). All the organs of mice exposed to cigarette smoke had modified pfc responses. The primary response in the lungs (Fig. 9) was virtually abrogated and although it was initiated in the spleen (Fig. 9) and lymph nodes (Fig. 9) it was reduced and short lived. After a second inoculation the response in the lungs of cigarette-smoke exposed mice was delayed and although a large number of pfc eventually developed there was a large participation of direct pfc. The secondary response in the lymph nodes and spleen was marked by a depression in the indirect pfc response.

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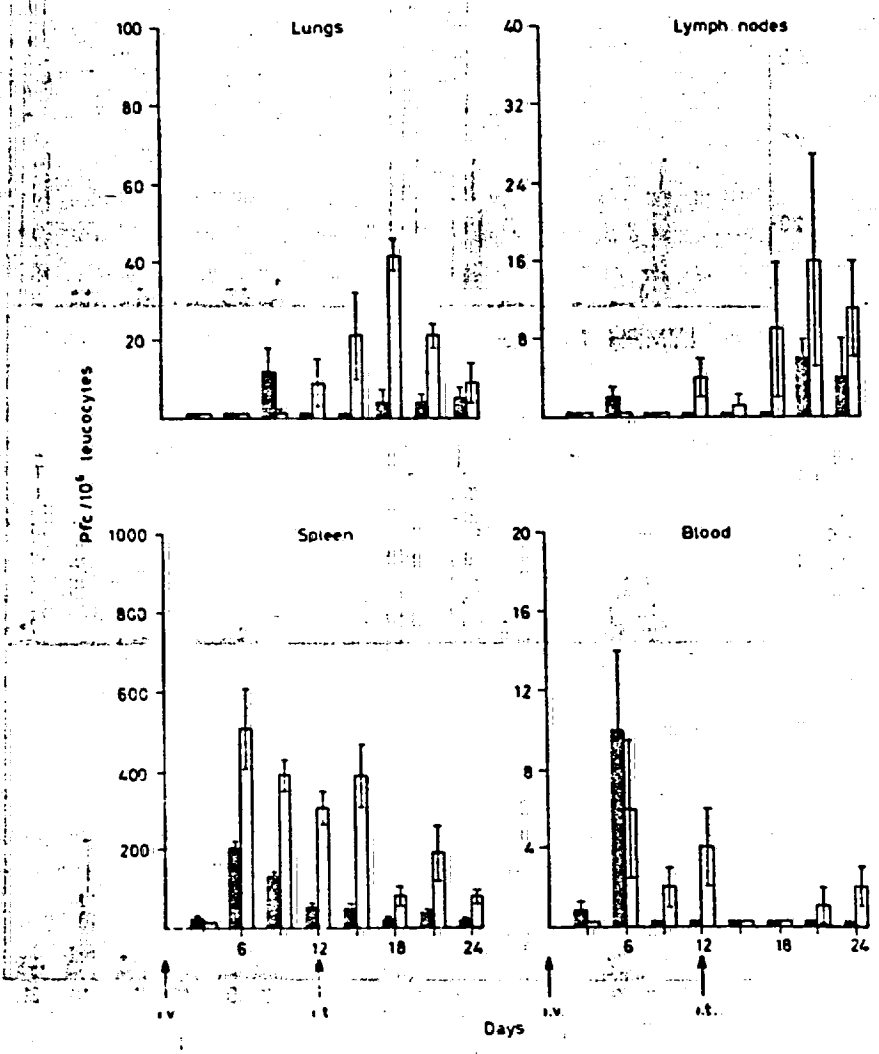

FIGURE 8 Plaque-forming-cell response in mice immunized with an intravenous (IV) then an intratracheal (IT) inoculation of 10^8 SRBC; direct pfc ■ , indirect pfc □ , ↑ indicates time of inoculation.

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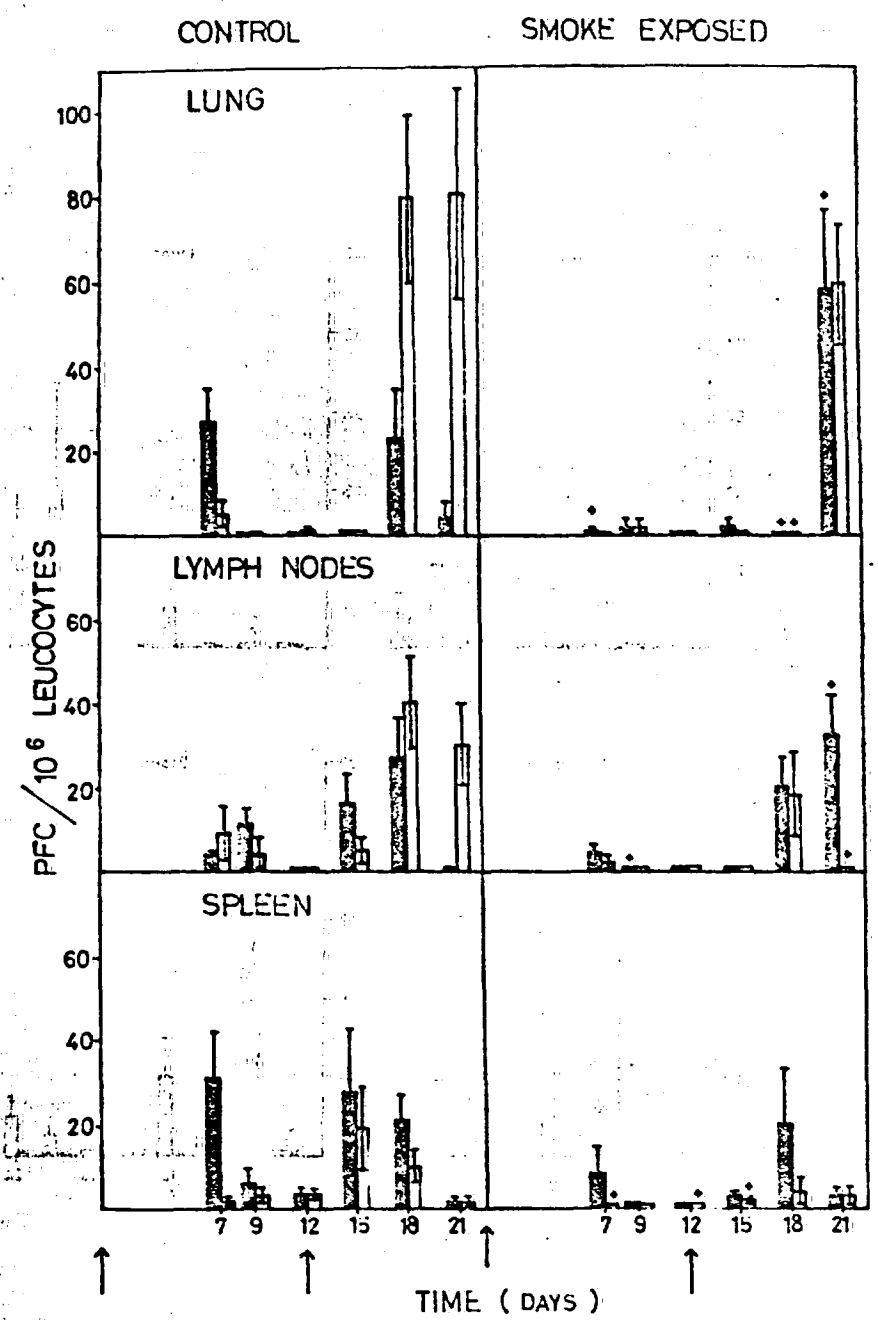

FIGURE 9 Primary and secondary plaque-forming-cell response in the spleen, lymph nodes and lungs of mice exposed to cigarette smoke for 26 weeks and control mice after intratracheal inoculations of SRBC. ↑ time of inoculation, direct pfc ■, indirect pfc □. Each result is the mean ± S.E. from groups of 5 mice. The significance of the differences between the cigarette smoke exposed and control mice was judged by the Student's t test: • $p<0.05$.

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(b) The development of the alterations in the primary immune response

The time course of the changes occurring in the primary immune response of the spleen, lungs and the pool of mediastinal and cervical lymph nodes was examined in C57Black mice exposed to the cigarette smoke for periods of up to 42 weeks. The direct and indirect pfc responses were determined in groups of mice 7 days after an intratracheal inoculation of 10^8 SRBC. The smoking regimes were continued throughout the immunization period. The primary responses of groups of five mice exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days were determined. The responses of the mice exposed to cigarette smoke, expressed as a percentage of the responses of age-matched-control mice, are shown in Figs. 10, 11 and 12. No significant change was found in the pfc responses of mice only exposed to the cigarette smoke after the inoculation of SRBC.

The influence of the exposure to cigarette smoke on the primary pfc response in the lung (Fig. 10) was apparent after 9 days. At this time the number of direct and indirect pfc detectable in the lungs fell to 30% of control values. After 182 days of smoke exposure the response in the lungs was less than 5% of the responses of control mice.

In the lymph nodes (Fig. 11) direct pfc response showed a rapid rise reaching a plateau between 96 and 120 days. This response then rapidly declined to show a severe depression by 182 days. The indirect pfc response in the lymph nodes was not stimulated to the same degree as the direct pfc response, but was depressed by prolonged exposure to cigarette smoke.

The direct pfc responses in the spleen became irregular after exposure to cigarette smoke, with many animals having high responses, and there was no consistent deviation from control levels until after 294 days smoking when a marked depression was apparent. The indirect pfc responses were similar, however the number of indirect pfc were low.

(c) Serum antibody

C57Black mice were exposed to cigarette smoke for 19 weeks and then inoculated intratracheally with 10^8 SRBC. The serum

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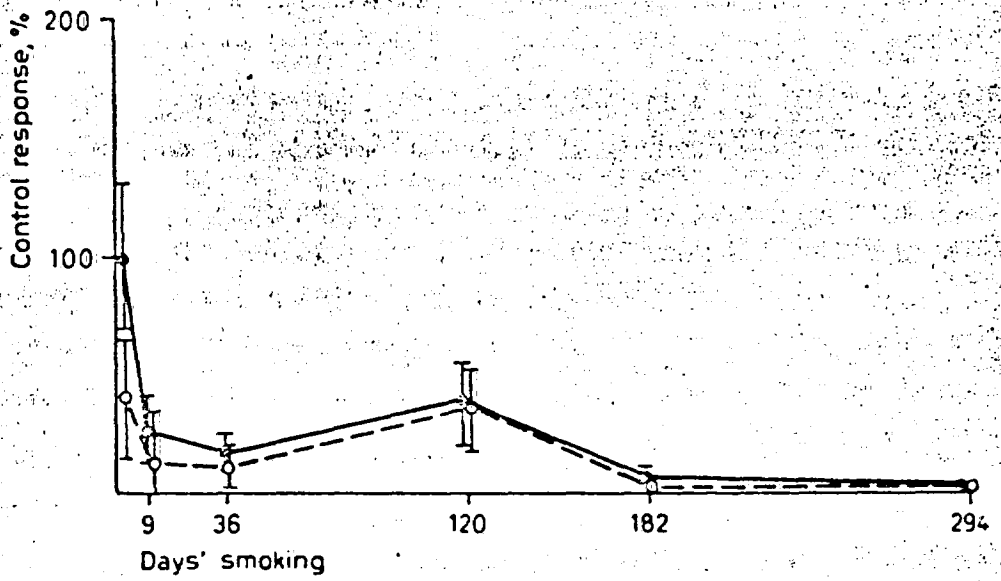

FIGURE 10 Effect of cigarette-smoke exposure on the plaque-forming-cell response in the lung to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct ● and indirect o, pfc. Control mice had an average of 22 direct and 13 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different direct and indirect pfc response ($p<0.05$) on days 9, 36, 120, 182 and 294.

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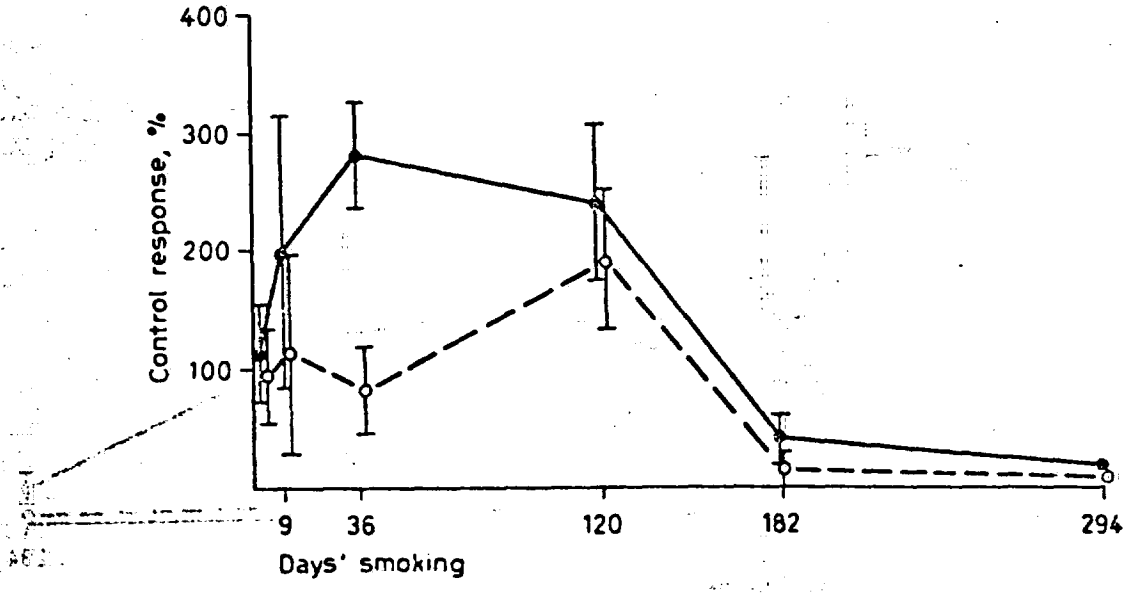

FIGURE 11 Effect of cigarette-smoke exposure on the plaque-forming cell response in the cervical and mediastinal lymph nodes to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct ●, and indirect o, pfc. Control mice had an average of 33 direct and 46 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different ($p<0.05$) direct pfc response on days 9, 182 and 294 and indirect response on day 294.

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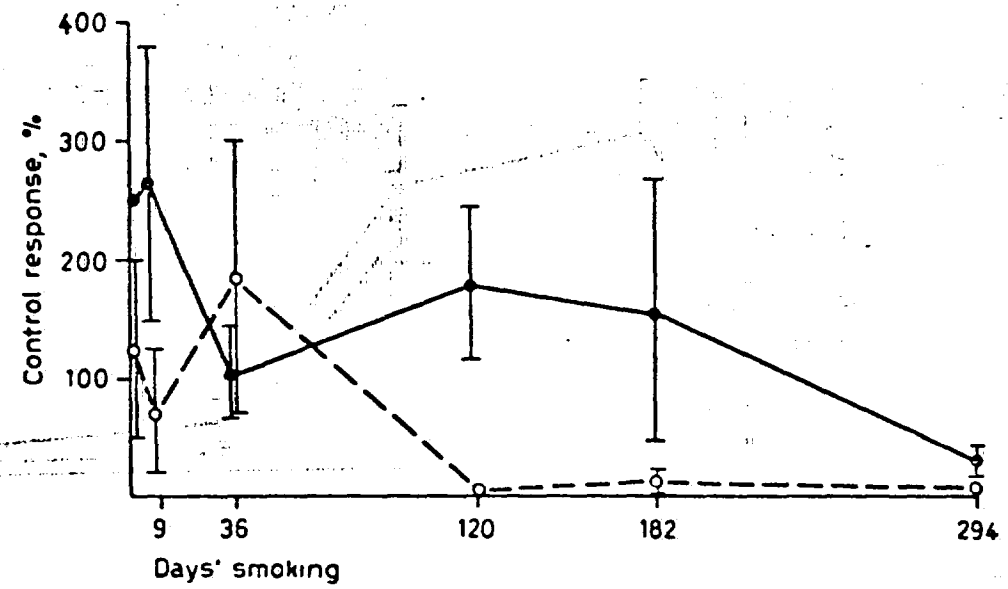

FIGURE 12 Effect of cigarette-smoke exposure on the plaque-forming cell response in the spleen to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct •, indirect o, pfc. Control mice had an average of 13 direct and 2 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different ($p<0.05$) direct pfc response on day 294.

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haemagglutinating and haemolytic antibody titres were measured 7 days after inoculation. The mice exposed to cigarette smoke had lower haemagglutinin and haemolysin titres (Table 11).

(d) Recovery of the pfc response after the cessation of cigarette smoking

C57Black mice were exposed to cigarette smoke for 42 weeks and then left for a further 16 weeks without cigarette smoke exposure. Their primary pfc response in the spleen, lung, and a pool of mediastinal and cervical lymph nodes was then measured 7 days after an intratracheal inoculation of 10^8 SRBC. Their response, expressed as a percentage of the response of age-matched-control mice, is shown in Table 12 compared to the response of mice exposed to cigarette smoke for 42 weeks. Recovery was evident in the direct pfc response in the lungs and lymph nodes. Mice discontinuing cigarette smoking still had low responses in the spleen. The indirect pfc response showed a similar trend but the numbers of indirect pfc in a primary response were low.

D. Humoral immune response of mice exposed to cigarette smoke to intraperitoneal inoculations of SRBC

Groups of C57Black mice were exposed to cigarette smoke for 1, 17 or 38 weeks, and their primary and secondary pfc and antibody responses to intraperitoneal inoculations of SRBC determined. The exposure to cigarette smoke was continued throughout the experiment. To examine the pfc and serum antibody responses to SRBC the mice were inoculated with 10^8 SRBC and where required a secondary response was elicited with another inoculation of 10^8 SREC on day 12. Groups of 5 mice were examined on day 4, 8, 16 and 20 after the first inoculation. The animals were exsanguinated and the serum stored at $-20^{\circ}C$ before assaying for antibody activity.

The spleen and a pool of cervical and mediastinal lymph nodes were examined for direct and indirect pfc. The pfc responses when considered per organ or per 10^6 leucocytes were similar. The direct and indirect pfc per 10^6 leucocytes in the spleens and lymph nodes are shown in Figs. 13 and 14.

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TABLE 11 Serum antibody titres of mice exposed to cigarette smoke for 19 weeks and inoculated intratracheally with SRBC

	Smoke exposed	Control
Haemolysin	0.22 ± 0.14	1.25 ± 0.45
Haemagglutinin	1.45 ± 0.24	3.4 ± 0.67

Each result is the mean ± S.E. of the $\log_2$ titre from 9 or 10 mice. The haemolysin and haemagglutinin titres of the mice exposed to cigarette smoke were significantly different from the titres of control mice as judged by Student's t test, $p<0.05$.

TABLE 12 Primary pfc responses of mice to an intratracheal inoculation of SRBC after discontinuing cigarette smoke exposure

	42 weeks smoke exposure		42 weeks smoke exposure and 16 weeks "recovery"	
	Direct pfc	Indirect pfc	Direct pfc	Indirect pfc
Lung	1.2 ± 0.3	0	** 75 ± 14.9	90 ± 58
Lymph nodes	11 ± 8.1	3 ± 3	* 69 ± 22.5	33.5± 15.2
Spleen	23 ± 8.0	0	(1) 41 ± 31.0	(2) 273 ± 256

The pfc responses expressed as a percentage of age matched controls. Except where indicated each result is the mean ± S.E. of the values from 5 mice: (1) 9 mice, (2) 6 mice. The significance of the differences between the smoke exposed group and the group discontinuing smoking was examined by Student's t test, * $p<0.05$, ** $p<0.001$

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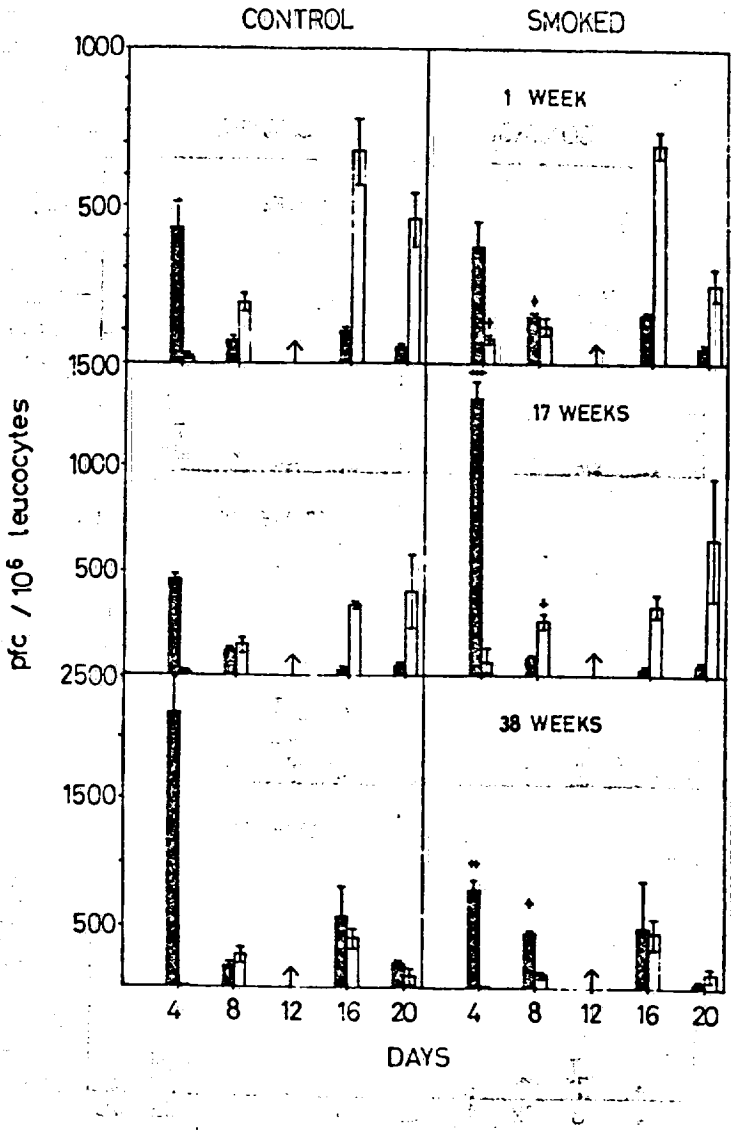

FIGURE 13 Primary and secondary pfc response in the spleen of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with two intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; direct pfc ■, indirect pfc □, ↑ inoculation. The significance of the difference between the cigarette smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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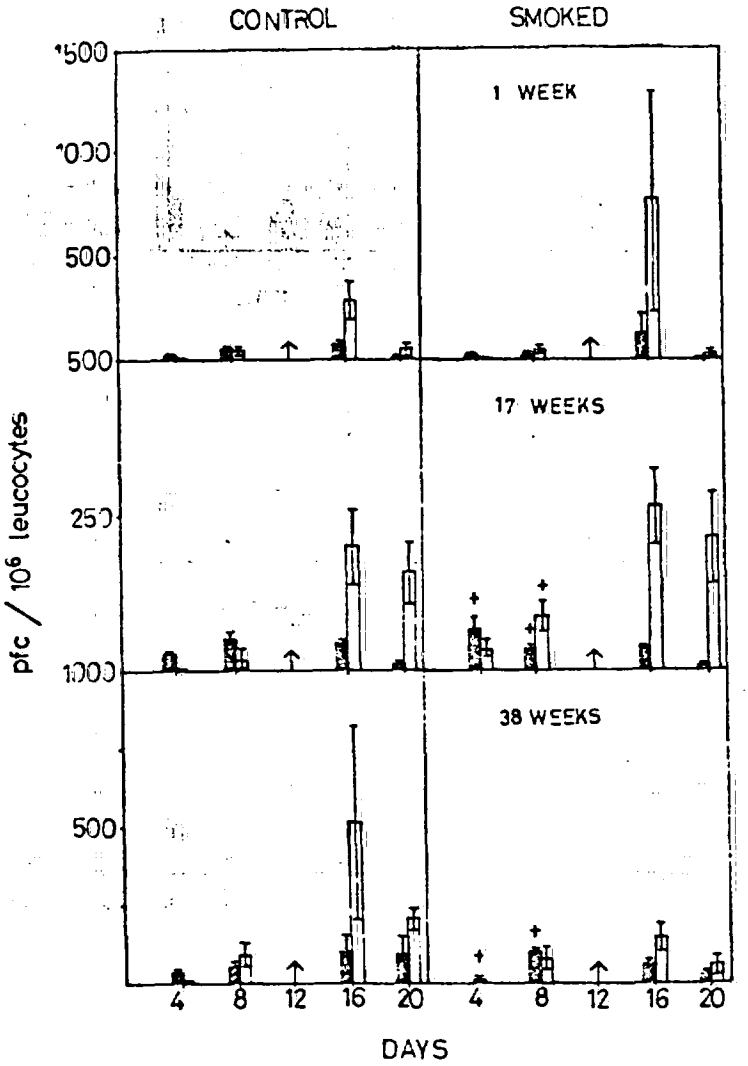

FIGURE 14 Primary and secondary pfc response in the mediastinal and cervical lymph nodes of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with two intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; direct pfc ■, indirect pfc □, † inoculation. The significance of the difference between the cigarette smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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After 1 week of smoke exposure the only differences between the smoke-exposed and control animals were that the animals exposed to cigarette smoke had a higher number of direct pfc in the spleen on day 8 and a higher number of indirect pfc in the spleen on day 4. Following 17 weeks of smoke exposure the mice had a highly elevated primary pfc response in the spleen and the lymph nodes. There was little difference between the secondary responses of the smoke-exposed and control animals.

The pfc response of the mice exposed to cigarette smoke for 38 weeks and the corresponding control mice had a high number of direct pfc in the spleen after the primary and secondary inoculation. However the mice exposed to cigarette smoke clearly had a depressed direct and indirect pfc response in the spleen after the primary inoculation. In the lymph nodes the primary pfc response of the mice exposed to cigarette smoke was delayed compared to the controls; the control mice had a significantly higher pfc response on day 4 but a significantly lower pfc response on day 8. No differences in the secondary responses were evident. The serum haemolytic and haemagglutinating antibody titres for the primary and secondary responses (Fig. 15) showed a stimulation of the primary haemolytic antibody response after 17 weeks exposure to cigarette smoke. A depression of the haemolytic antibody response after the primary and secondary inoculations was evident in mice exposed to cigarette smoke for 38 weeks.

E. Natural antibody producing cells to SRBC in mice exposed to cigarette smoke

A group of C57Black mice was exposed to cigarette smoke for 16 weeks and then the cervical and mediastinal lymph nodes and the spleen examined for 'natural' pfc to SRBC. Corresponding unimmunized control animals were also examined. No significant difference in the numbers of pfc in control mice and mice exposed to cigarette smoke was evident (Table 13).

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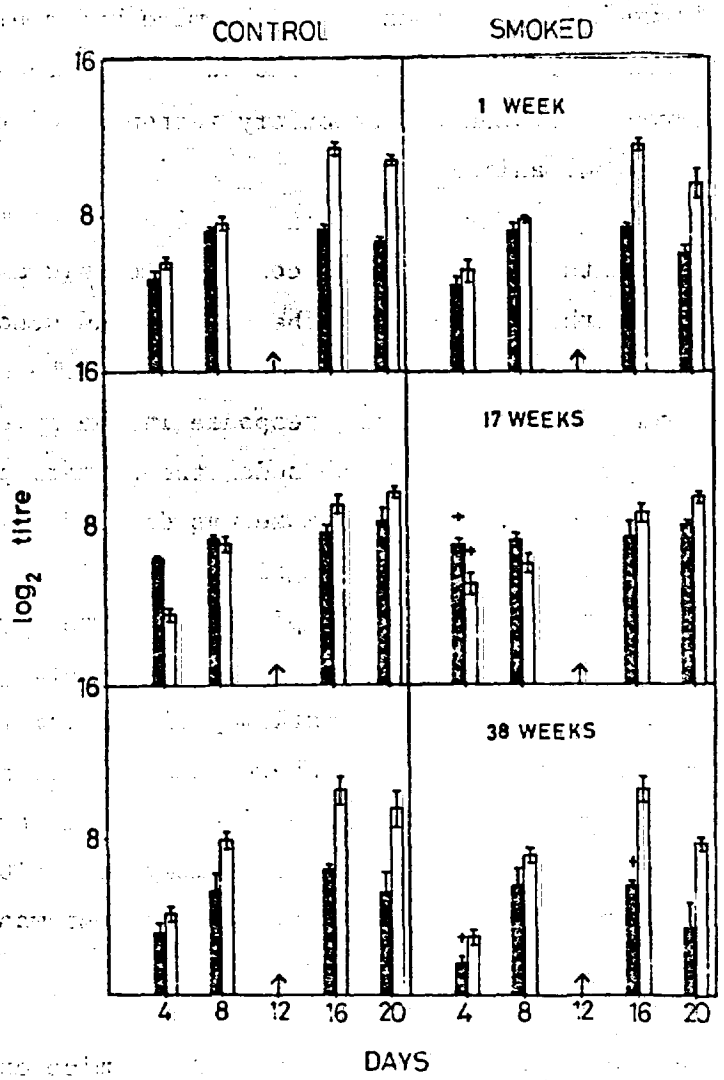

FIGURE 15 Primary and secondary serum antibody response of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; haemolysin ■, haemagglutinin □, ↑ inoculation. The significance of the differences between the smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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TABLE 13 Plaque forming cells to SRBC in unimmunized mice exposed to cigarette smoke for 16 weeks

	pfc/10^6 leucocytes			
	Smoke exposed		Control	
	Direct pfc	Indirect pfc	Direct pfc	Indirect pfc
Spleen	0.2 ± 0.2	0	0.8 ± 0.4	0.2 ± 0.29
Lymph nodes	0	0	0.2 ± 0.2	0.2 ± 0.2

Each result is the mean ± S.E. of the values from 5 mice. As judged by the Student's t test there was no significant difference between the cigarette-smoke exposed and control mice. Mice were exposed to cigarette smoke for 16 weeks and then the 'natural' number of pfc in the spleens and pool of mediastinal and cervical lymph nodes was determined.

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F. The immune response of mice exposed to cigarette smoke to polyvinylpyrrolidone (PVP)

Groups of C57Black mice were exposed to cigarette smoke for 8, 15, 30 and 41 weeks. The mice were then inoculated intravenously with 0.2µg of PVP (MW 360,000) in 0.1ml of PBS. One week after this inoculation the serum-antibody titres were measured by the indirect haemolysis of PVP coated SRBC. The exposure to cigarette smoke was continued throughout all inoculation regimes. The indirect-haemolytic-antibody titres (Table 14) did not show any significant difference between mice exposed to cigarette smoke and control animals. The serum of normal mice and unimmunized mice exposed to cigarette smoke for 15 weeks did not have a detectable titre to PVP.

G. The effect of cigarette-smoke solutions on antibody-producing cells and antibody activity *in vitro*

(a) Antibody-forming cells

Plaque-forming cell preparations were prepared from the spleens of mice, 4 - 6 days after an intraperitoneal inoculation of 10^8 SRBC. The cell preparations were incubated in final dilutions of 1/2.5, 1/10 and 1/100 of cigarette-smoke solution and incubated for 0, 1, 2 and 4 hours at 37°C before assaying for pfc. Except for the assay performed immediately after the addition of smoke solution, the cells were centrifuged (200g, 5 minutes) and resuspended in fresh medium before the pfc assay. The cigarette-smoke solution had no immediate effect on the number of pfc (Fig. 16), but after continued incubation the number of pfc showed a transient depression. The extent of the inhibition and rate of recovery was dependent on the concentration of smoke solution. A further decrease rather than a recovery of pfc numbers was found if cells that had been incubated in a 1/10 smoke solution for 1 hour were retreated with a fresh 1/10 smoke solution for another hour.

(b) Antibody activity

The effect of cigarette-smoke solutions on the antibody activity of 7S and 19S fractions of rabbit anti-SRBC serum prepared

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TABLE 14 Serum antibody response of mice chronically exposed to cigarette smoke to polyvinylpyrrolidone

Weeks exposure[1]	Log_2 Antibody titre	
	Smoke exposed	Control
8	2.6 ± 0.40	1.4 ± 0.51
15	2.8 ± 0.20	2.4 ± 0.24
30	[2] 3.0 ± 0.45	[2] 3.3 ± 0.21
41	[3] 2.6 ± 0.34	[3] 2.0 ± 0.71

Each result is the mean ± S.E. of log_2 antibody-titre in the serum one week after the inoculation of PVP. Each group had 5 mice except where indicated: (2) 6 mice, (3) 3 mice. (1) Weeks exposure to cigarette smoke before the inoculation of PVP.
No significant difference between cigarette smoke exposed and control animals was found as judged by Student's t test.

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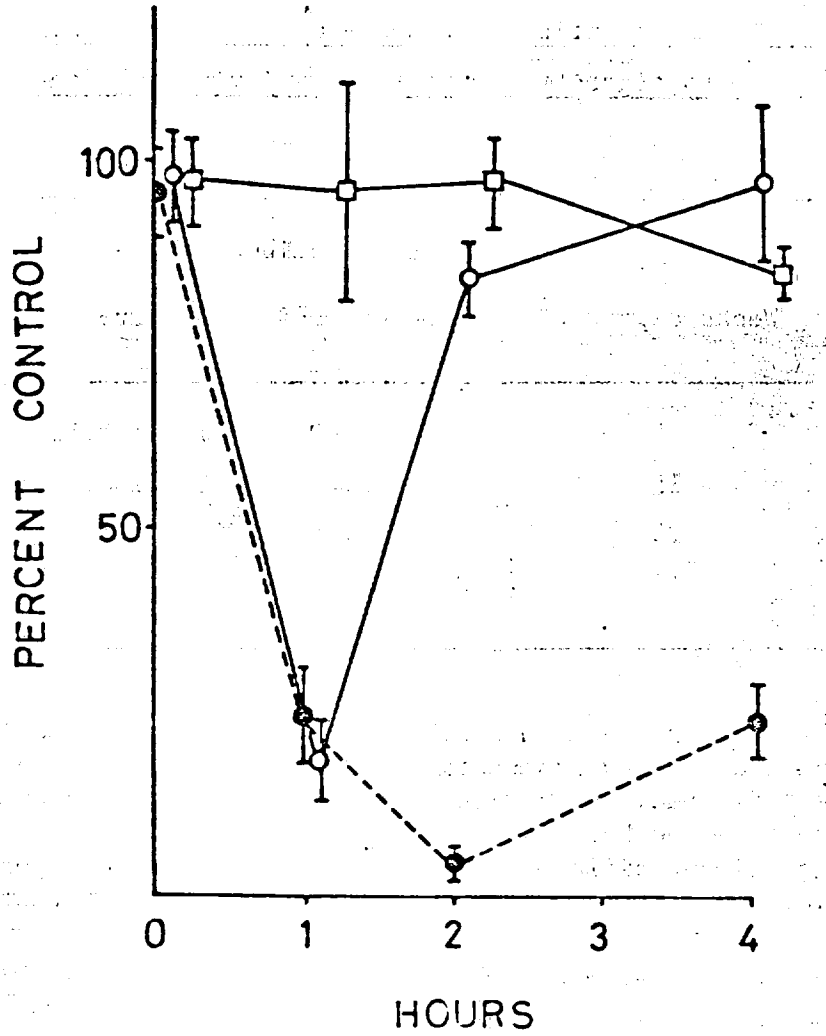

FIGURE 16 Effect of cigarette-smoke solutions on plaque-forming cells *in vitro*. Each result is the mean ± S.E. of 6 cultures. The results show the number of pfc found in cultures of antibody-forming cells, when assayed at 0, 1, 2 and 4 hours after the addition of smoke solution to the cultures, as a percentage of control values. Three dilutions of smoke solution were used; ● 1/2.5, o 1/10, □ 1/100.

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by Sephadex gel filtration, was examined. Haemagglutinating and haemolytic antibody activity was studied in both fractions. The fractions were incubated at 37°C in a final concentration of 1/2.5 of a fresh stock smoke solution for 1 hour. More fresh smoke solution was then added to a final concentration of 1/2.5 of the fresh smoke solution and the fractions incubated for another hour. The haemagglutinating and haemolytic antibody titres were measured after the second hour and compared to controls (Table 15). The smoke solutions did not affect the titres. The addition of smoke solution to titrations using reagents not preincubated with smoke solutions did not affect the titres.

H. Discussion

(a) Humoral response of normal mice to local and systemic inoculation of SRBC

After an intratracheal inoculation of SRBC, pfc were found in the lungs and regional lymph nodes, the spleen and in the peripheral blood. However the axillary lymph nodes, did not respond significantly to the intratracheal inoculation. Pfc could be found in the lungs and lymph nodes after either topical or systemic immunization but generally the response in the respiratory system was greatest after local immunization.

It is important to consider whether or not the pfc detected in the lungs were part of the population circulating in the blood. This was clearly not the case where the immunization regime produced little or no response in the blood, but the interpretation was not so obvious when large responses occurred in both organs. In many of the cases where large responses were found in the blood, the concentration of pfc (per 10^6 leucocytes) in the lung was less than 10% of the concentration in the blood. If this percentage was taken as the contribution from freely circulating cells, the remaining pfc found in the lung must have had some closer association with the respiratory system.

Following two intravenous inoculations of SRBC, large numbers of pfc were found in the blood and the lung. The responses in these organs were highly correlated. In the lung, the number of pfc/10^6 leucocytes reached 50% of that found in the blood. As

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TABLE 15 Effect of cigarette-smoke solutions on antibody activity

	Smoke exposed		Control	
Fraction	Haemagglutinin	Haemolysin	Haemagglutinin	Haemolysin
19S	2.3 ± 0.3	6 ± 0	2.3 ± 0.3	6 ± 0
7S	2.0 ± 0	2.0 ± 0	2.0 ± 0	2 ± 0

Each result is the mean ± S.E. of the result of 3 titrations of the antibody activity in fractions exposed to cigarette smoke solutions for 2 hours.

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previously discussed, only a small proportion of these could be accounted for in terms of transient circulating cells. It therefore appeared that the majority of pfc in the lungs were seeded from the circulation, or had been initiated locally. Hiramoto, Habeeb and Hamlin (1970), and Hiramoto and Hamlin (1970) have reported that the lung could be an extralymphoid site of antibody production after systemic immunization. The antibody-forming cells were found to accumulate around the pulmonary vessels and alveolar walls (Marshall and White 1950; Hiramoto, Habeeb and Hamlin 1970). In contrast to the lung, the mediastinal and cervical lymph nodes responded to systemic immunization but this response did not appear to be associated with the pfc found in the blood.

Intratracheal inoculation of SRBC produced a response in mice which resulted in pfc being detected in the blood, spleen, lung and the mediastinal and cervical lymph nodes. After this inoculation about 200 pfc/lymph node were produced. Only a low number were found in the blood. It was difficult to estimate the total number of cells in the lung but in the order of 100 pfc/lung were probably produced. The lung, the lymph nodes and the spleen showed a secondary response to a second intratracheal inoculation. The quantitative and temporal aspects of the response in the lung indicated that it was locally produced. An intratracheal inoculation of SRBC has been found to induce a local antibody response in canine tracheal-pouches (Lieberman *et al* 1972). After local immunization with SRBC, pfc have also been found in the endobronchial lavages of rabbits (Holub and Hauser 1969; Ford and Kuhn 1973a,b).

When the local response induced by intratracheal immunization was boosted by a systemic inoculation, large responses were detected in the lungs and the lymph nodes as well as a large number of pfc in the blood. The response in the lung had a higher concentration of pfc than the blood and the responses were not significantly correlated. The intravenous inoculation apparently boosted the response centralised in the lungs and lymph nodes. However seeding from the spleen cannot be eliminated. Mice preimmunized systemically responded to the local inoculation by primarily producing an indirect pfc response, little greater in magnitude to the primary response to a local inoculation.

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The intratracheal inoculation of SRBC produced a pfc response which appeared to be localised in the lung and in the lymph nodes and spleen, and provided a method for assessing the immune response in the respiratory tract.

(b) The effect of exposure to cigarette smoke on the humoral immune response

The humoral immune responses of mice exposed to cigarette smoke to intratracheal and intraperitoneal inoculations were measured. The exposure to cigarette smoke produced almost identical alterations to the response to locally or systemically administered SRBC. Cigarette-smoke exposures for up to 17 weeks stimulated the primary pfc responses in the spleen and lymph nodes to both inoculation regimes. The statistical significance of the stimulation of the primary pfc response in the spleen after intratracheal inoculation has been established (Nulsen, Holt and Keast 1974). After continued exposures to cigarette smoke the primary immune response to locally and systemically inoculated SRBC was depressed. These included the primary pfc responses in the lung, lymph nodes and spleen as well as serum antibody; both haemagglutinating and haemolytic antibody titres elicited after either local or systemic inoculation were decreased. The depression of the pfc response in the lungs occurred soon after the commencement of smoke exposure and a phase of enhanced response was not detected. However mice inoculated intratracheally with SRBC and then exposed to cigarette smoke did not have depressed pfc responses. The only alteration in the pfc and antibody responses of mice to systemic inoculation of SRBC, produced by one week of exposure to cigarette smoke, was a slight increase in the pfc response of the spleen. Mice chronically exposed to cigarette smoke could at least partially recover their ability to respond to SRBC when the exposure to cigarette smoke was discontinued.

As the primary responses of mice exposed to cigarette smoke and control mice were different, the effect of a second inoculation of immunogen can not be strictly compared. Even so, it appeared that mice exposed to cigarette smoke were able to respond normally to continued antigenic challenge. The secondary pfc responses to intraperitoneal inoculations of SRBC were almost identical in control mice and mice exposed to cigarette smoke. This occurred regardless of whether the primary response of the mice exposed to

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cigarette smoke was stimulated or depressed. The secondary pfc responses of mice exposed to cigarette smoke, to intratracheal inoculations of SRBC, were still lower than the responses of control mice and had an abnormally large participation of direct pfc. However the responses after a second intratracheal inoculation were considerable higher than the primary responses and it appears probable that the difference in the effect of exposure to cigarette smoke on the secondary intratracheal and intraperitoneal responses was only due to the ability of the intraperitoneal inoculations to elicit a more vigorous immune response. It should be noted that a small decrease in the serum antibody response to a second intraperitoneal inoculation was found in mice chronically exposed to cigarette smoke.

As SRBC have been found to be T-cell dependent immunogens, (Davies *et al* 1971; Playfair 1971) serum antibody responses to an immunogen not requiring T-cells, PVP (Andersson and Blomgren 1971), was also measured. No significant difference could be found between the response of control mice and mice exposed to cigarette smoke for periods up to 41 weeks. This does not necessarily imply that either T-cell function is impaired or B-cell function is not impaired in mice exposed to cigarette smoke. The mice exposed to cigarette smoke could have a defect in macrophage function; there is evidence showing that macrophages are required for lymphoid cell co-operation (Feldman 1972; Waldron, Horn and Rosenthal 1973). It is also possible that a defect in T-cell and B-cell co-operation could be caused by a defect of B-cells. However the response to PVP and the secondary responses to SRBC indicate that it was possible to stimulate a normal humoral immune response in cigarette-smoke-exposed mice.

Experiments with solutions of cigarette smoke showed that haemagglutinating and haemolytic activities of 19S and 7S antibodies were resistant to the acute effects of cigarette smoke. It would, however, be reasonable to expect that cigarette smoke could have a direct inhibitory action on the function of antibody-forming cells in the lung. Since human cigarette smokers typically smoke several cigarettes daily, their respiratory tract would be subject to the direct effects of cigarette smoke for a considerable period of time. The exposure to cigarette smoke did not affect the antibody-producing cell response in the lungs of mice exposed to cigarette smoke after

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immunization. However, the mice in these experiments were not exposed to cigarette smoke on the day of assay.

SECTION 2 CELL-MEDIATED IMMUNITY

A. Introduction

Cell-mediated immune reactions result from the action of antigen on T-cells which then respond by blast transformation, proliferation and the production of substances which participate in the immune reaction (Bellanti 1971; World Health Organization 1973). These substances may be effector molecules acting on tumour cells and infectious agents, or mediator molecules, which recruit other cells into the immune reaction. The major collaborating cells in cell-mediated immune reactions appear to be the mononuclear phagocytes. A large number of biological activities of products from activated lymphocytes have been reported and at least several different molecules are responsible for the activities (David 1971; World Health Organization 1973). The incorporation of H^3-thymidine into lymphocytes in the presence of PHA is a measure of the proliferation of T-cells and has been recognized as an *in vitro* correlate of the potential to mount cell-mediated immune reactions (Rodey and Good 1969; Adler *et al* 1970; Bellanti 1971; Keast and Bartholomaeus 1972; Stobo, Rosenthal and Paul 1972; World Health Organization 1973). The potential of mice exposed to cigarette smoke to elicit cell-mediated immune reactions was estimated by measuring the incorporation of H^3-thymidine into lymphocytes incubated with PHA.

B. Response of lymphocytes from mice exposed to cigarette smoke to PHA

Groups of C57Black mice were exposed to cigarette smoke for 5, 15, 25 and 35 weeks. The responsiveness of the lymphocytes from the spleen, peripheral blood and a pool of the cervical and mediastinal lymph nodes of individual mice to PHA was determined by a micromethod measuring the incorporation of H^3-thymidine into the DNA of lymphocytes incubated with various concentrations of PHA (Keast and Bartholomaeus 1972). Tests on the lymphocytes from 6 - 12

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week old and 40 week old controls were also performed. Triplicate cultures at each PHA concentration were set up and the means and S.E. for groups of three animals at each exposure time were determined.

In the experiments, the responsiveness of the lymphocytes recovered from the blood and spleen to PHA exhibited similar changes throughout the period of cigarette smoke exposure (Figs. 17, 18). After five weeks exposure to cigarette smoke the responsiveness of the lymphocytes from both these organs was depressed. At weeks 15 and 25 a transient recovery of responsiveness was noted. By the 35th week of exposure the peak response of the lymphocytes from each organ was depressed. However, only the peak response of lymphocytes from blood was significantly depressed below the peak response of the lymphocytes from 40 week old controls (Fig. 17). There was a tendency for the splenic lymphocytes to respond maximally to higher levels of PHA than the 40 week old controls.

The lymph node cells displayed an enhanced response to PHA after 5 weeks cigarette smoke exposure but after 15 weeks the lymphocytes would only respond to high concentrations of PHA. A large response was obtained with the lymph node lymphocytes from mice exposed for 25 weeks but after 35 weeks exposure to cigarette smoke responsiveness to PHA was markedly depressed. The peak response of the lymph node lymphocytes from the mice exposed for 35 weeks was significantly lower than the response of the lymph node lymphocytes from the 40 week control mice. Hence the ultimate result of the prolonged cigarette smoke exposure was a depression of the responsiveness of lymphocytes to PHA, particularly in the lymph nodes draining the respiratory system (Fig. 19).

C. Discussion

The incorporation of H^3-6-thymidine into lymphocytes in the presence of PHA is a measure of the proliferation of T-cells (Rodey and Good 1969; Adler *et al* 1970; Bellanti 1972; Keast and Bartholomaeus 1972; Stobo, Rosenthal and Paul 1972; World Health Organization 1973). This is a function of the number of T-cells as well as their ability to divide after contact with the mitogen. T-cells form the major proportion of the recirculating lymphocytes and procedures which deplete recirculating lymphocytes diminish cell-mediated immune

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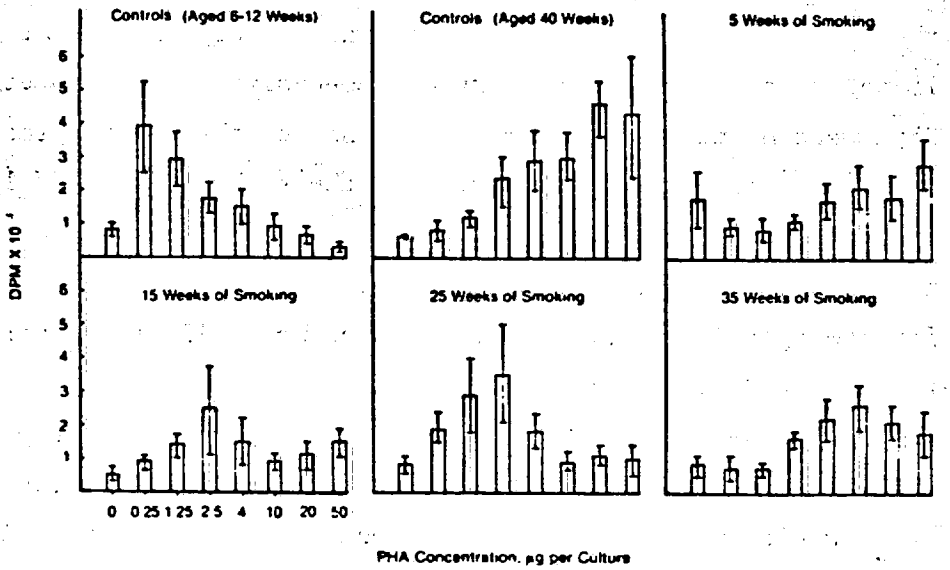

FIGURE 17 The PHA response of peripheral blood lymphocytes. Each point represents the mean ± S.E. derived from the cultures from three mice. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. The peak response after 35 weeks smoking was significantly different from that of control mice as determined by Student's t test ($p<0.05$).

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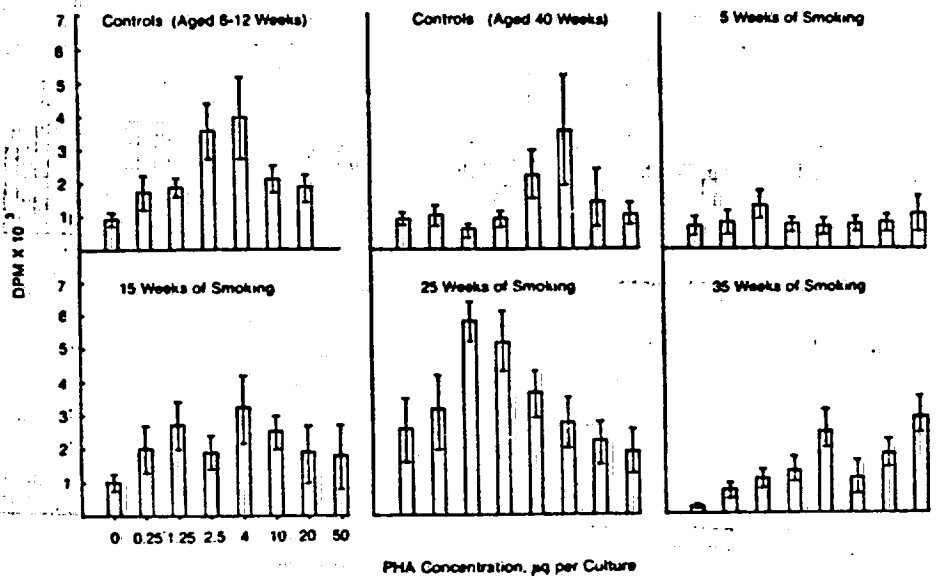

FIGURE 18 The PHA response of splenic lymphocytes. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. Each result represents the mean ± S.E. derived from cultures from three mice. The peak response after 35 weeks smoking was not significantly different from that of control mice as determined by Student's t test.

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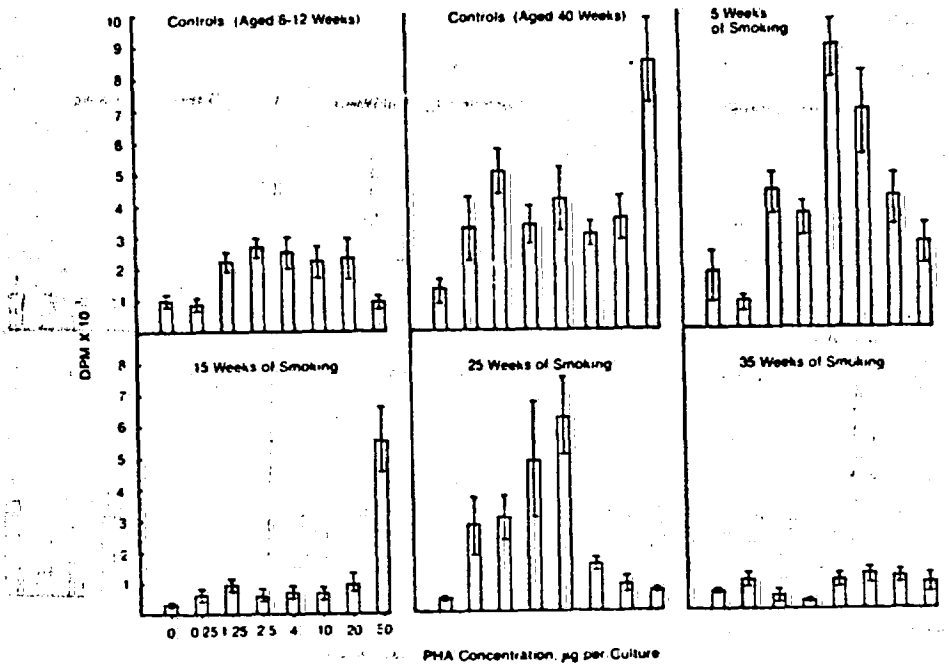

FIGURE 19 The PHA response of cervical and mediastinal lymph node lymphocytes. Each result represents the mean ± S.E. derived from cultures from three mice. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. The peak response after 35 weeks smoking was significantly different from that in control mice as determined by Student's t test ($p<0.01$).

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reactions (World Health Organization 1970; Gowans 1970; Davies *et al* 1971). However as cell-mediated immune reactions can be elicited in the lung without sensitizing the cells in the spleen it appears that the T-cell population throughout the body is not homogeneous (Henney and Waldman 1970). The circulating nature of the PHA-responsive cell is probably demonstrated by the similarity of the changes occurring in the responsiveness of cells of the spleen and peripheral blood to PHA produced by the inhalation of cigarette smoke. The changes in the responses of the lymphocytes from the cervical and mediastinal lymph nodes to PHA, with one major exception, were also similar to the responses of the lymphocytes from the spleen and peripheral blood. After 5 weeks exposure to cigarette smoke the responsiveness of the lymphocytes to PHA from the lymph nodes was enhanced while the responsiveness of the lymphocytes from the blood and spleen was depressed. However, chronic exposure to the cigarette smoke eventually diminished the responsiveness of the lymphocytes in the blood and lymph nodes to PHA. Although the depression of the responsiveness in the spleen was not statistically significant, the tendency of the lymphocytes to respond maximally to higher concentrations of PHA may indicate an impairment. In immunological deficiency syndromes, both the pattern of the PHA dose-response curve and the magnitude of the peak response is altered from normal limits (Hosking, Fitzgerald and Simons 1971).

SECTION 3 PHAGOCYTIC FUNCTION

A. Introduction

The investigations of phagocytic function in mice exposed to cigarette smoke were designed to examine: (i) the ability of the alveolar macrophages to kill bacteria; (ii) the ability of the liver, spleen and lung to remove and process bacteria from the blood.

The phagocytic activity of the alveolar macrophages was determined by measuring the inactivation and clearance of intratracheally inoculated radiolabelled *Pseudomonas aeruginosa*. The inactivation of the bacteria in the lung has been shown to be performed by alveolar macrophages (Green and Kass 1964a,b).

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It has been well established that exposure of macrophages *in vitro* to cigarette smoke diminishes their phagocytic activity (Green and Carolin 1967; Green 1968; Lentz and DiLuzio 1972, 1973). Experiments examining the effect of cigarette smoke exposure on macrophages *in vitro* were performed with emphasis placed on the ability of macrophages exposed to cigarette smoke to regain their phagocytic activity.

B. Pulmonary bacteriocidal activity

Viable, radiolabelled *Pseudomonas aeruginosa* were inoculated intratracheally into female mice which had been exposed to cigarette smoke for 18 or 34 weeks and male mice which were exposed to cigarette smoke for 37 weeks. After 5 and 24 hours the viability and radioactivity of the bacteria inoculated into the lungs of groups of 3 mice were determined. The results (Table 16) are shown as a percentage of values obtained immediately after inoculation. The ratio of the viable bacterial count to the radioactivity is shown as an indication of the viability of the bacteria not cleared from the lungs.

The radioactivity and bacterial counts in the lungs of the female mice exposed to cigarette smoke for 18 weeks were not significantly different from the values of control mice. The female mice exposed to cigarette smoke for 34 weeks had a higher number of viable bacteria remaining in their lungs 24 hours after the inoculation. The male mice exposed to cigarette smoke for 37 weeks had an increased number of viable bacteria remaining in the lung 5 hours after the inoculation. Both the number of viable bacteria and the radioactivity in the lungs of these mice at 24 hours was higher than control mice. The ratio of the bacterial viability and radioactivity in the lung show that the killing of the bacteria not cleared from the lungs was depressed.

C. Blood clearance

Radiolabelled *Pseudomonas aeruginosa* were inoculated intravenously into female mice exposed to cigarette smoke for 18 and 34 weeks and male mice exposed for 37 weeks. The phagocytic indices for their clearance from the blood are shown in Table 17. There was no

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TABLE 16 Pulmonary bacteriocidal and clearance activity of cigarette-smoke-exposed mice

Group[1]		Time[2]	DPM/lung[3]	Bact/lung[4]	Bact/lung / DPM/lung[5]
18 week (female)	Control	0	100.0 ± 13[6]	100.0 ± 8.0[6]	100.0 ± 6.7[6]
	Smoker	5	65.2 ± 6.1	16.4 ± 7.5	28.0 ± 15.0
	Control	5	65.5 ± 15.0	10.0 ± 1.2	16.0 ± 2.3
	Smoker	24	18.4 ± 0.16	0.23 ± 0.057	1.2 ± 0.57
	Control	24	18.7 ± 5.5	0.05 ± 0.0057	0.2 ± 0.04
34 week (female)	Control	0	100.0 ± 22.0	100.0 ± 26.0	100.0 ± 28.0
	Smoker	5	53.0 ± 3.8	8.0 ± 0.57	15.0 ± 1.4
	Control	5	38.0 ± 5.5	8.8 ± 0.63	25.0 ± 0.8
	Smoker	24	12.7 ± 1.0	0.24 ± 0.00057*	2.0 ± 0.17*
	Control	24	13.0 ± 1.0	0.06 ± 0.023	0.4 ± 0.17
37 week (male)	Control	0	100.0 ± 5.8	100.0 ± 13.0	100.0 ± 18.0
	Smoker	5	88.0 ± 5.5	75.0 ± 11.0*	85.0 ± 8.1
	Control	5	61.2 ± 9.4	23.0 ± 4.6	37.0 ± 1.5
	Smoker	24	22.4 ± 1.5*	0.65 ± 0.0034**	4.7 ± 1.1*
	Control	24	11.6 ± 3.1	0.038± 0.0075	0.38 ± 0.17

The significance of the differences between the controls and the smokers was determined by Student's t test: * $p<0.05$, ** $p<0.01$.

(1) Weeks exposure of the smokers to cigarette smoke (sex).

(2) Time after the administration of bacteria to the respiratory tract.

(3) Disintegrations per minute per lung as a percentage of the value obtained immediately after inoculation.

(4) Viable bacteria per lung as a percentage of the value obtained immediately after inoculation.

(5) $\frac{\text{Bacteria/lung (as \% bacteria/lung at time 0)}}{\text{DPM/lung (as \% DPM/lung at time 0)}} \times 10^2$.

(6) Each result is the mean ± S.E. of the values obtained from groups of three mice.

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TABLE 17 Phagocytic indices of the clearance of *Pseudomonas aeruginosa* from the blood of cigarette-smoke-exposed mice

Group	Smoke-exposed	Controls
18 week[1], female	0.075 ± 0.0063[2]	0.075 ± 0.0063
34 week, female	0.089 ± 0.0036	0.089 ± 0.0036
34 week, male	0.050 ± 0.0030	0.051 ± 0.0033

No significant differences between the smoke exposed and control groups were found as determined by Student's t test.

(1) Weeks exposed to fresh cigarette smoke.

(2) Each result is the mean ± S.E. of the phagocytic indices of a group of 6 mice.

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detectable difference between the values of mice exposed to cigarette smoke and corresponding control mice. The radioactivity in the liver, spleen, lungs and kidneys at 10 and 180 minutes after the inoculation of the bacteria is shown in Tables 18 - 20, as a percentage of the inoculum. The radioactivity per gram in each organ, of both mice exposed to cigarette smoke and control mice, was higher than the radioactivity per ml of blood. The mice exposed to cigarette smoke for 34 or 37 weeks had considerably less radioactivity than controls in the liver at 10 minutes and the amount of radiolabel eliminated from the livers between 10 and 180 minutes was also significantly depressed. The amount of radiolabel in the kidneys of mice exposed to cigarette smoke for 18 and 34 weeks was significantly higher than in control mice.

D. Phagocytosis *in vitro*

Cigarette-smoke exposure produced a dose-dependent decrease in the viability and adherence of the macrophages (Fig. 20). The phagocytic activity of the remaining macrophages, as measured over 2 hours, was depressed (Fig. 20). Controls were sham treated. This decrease in phagocytosis was observed if the macrophages were incubated in both high and low concentrations of bacteria (Fig. 21). Phagocytosis was also inhibited when the macrophages were exposed to the vapour phase of cigarette smoke (Table 21). To examine the recovery from the exposure to cigarette smoke the macrophages were exposed to one puff of cigarette smoke and viability and phagocytic activity measured 30 minutes or 24 hours after the smoke exposure. To measure the phagocytic activity of the macrophages in these experiments the macrophages were incubated with bacteria for 2 hours. The experiment was also conducted using macrophages exposed to the vapour phase and macrophages which were exposed to cigarette smoke and immediately washed three times with PBS. The washing was performed by gently filling the petri dishes with 1ml of PBS and immediately removing the PBS. The phagocytic activity in each exposure group was still less than the controls after 24 hours (Table 21). However at 24 hours the macrophages exposed to whole smoke and washed or macrophages only exposed to the vapour phase had a higher phagocytic activity than macrophages exposed to whole smoke.

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TABLE 18 The distribution of radioactivity in female mice exposed to cigarette smoke for 18 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ[1] 10 mins	180 mins
Liver	Control	30.6 ± 1.8	14.2 ± 0.30
	Smoker	34.3 ± 3.6	16.6 ± 0.86
Lung	Control	3.8 ± 0.32	1.5 ± 0.12
	Smoker	3.5 ± 0.22	1.7 ± 0.18
Spleen	Control	1.1 ± 0.063	1.2 ± 0.075
	Smoker	1.4 ± 0.13	1.6 ± 0.13
Kidney	Control	4.0 ± 0.29	3.8 ± 0.098 **
	Smoker	6.7 ± 0.12 ‡	4.1 ± 0.11
Blood[2]	Control	6.2 ± 0.57	3.1 ± 0.17
	Smoker	6.7 ± 0.46	3.5 ± 0.34

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: ‡ $p<0.01$.

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: ** $p<0.01$.

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TABLE 19 The distribution of radioactivity in female mice exposed to cigarette smoke for 34 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ(1) 10 mins	180 mins
Liver	Control	+34.2 ± 1.5	*10.8 ± 1.6
	Smoker	21.6 ± 4.5	13.2 ± 0.40
Lung	Control	1.8 ± 0.18	1.1 ± 0.11
	Smoker	1.6 ± 0.091	1.2 ± 0.057
Spleen	Control	1.2 ± 0.057	0.78 ± 0.14
	Smoker	1.0 ± 0.05	0.76 ± 0.20
Kidney	Control	+4.6 ± 0.30	3.5 ± 0.11
	Smoker	5.7 ± 0.19	3.9 ± 0.18
Blood(2)	Control	3.9 ± 0.44	1.2 ± 0.023
	Smoker	4.0 ± 0.39	0.92 ± 0.063

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: + $p<0.05$,

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: * $p<0.05$,

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TABLE 20 The distribution of radioactivity in male mice exposed to cigarette smoke for 37 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ (1) 10 mins	180 mins
Liver	Control	+47.8 ± 2.5	*15.8 ± 2.1
	Smoker	32.2 ± 3.4	15.5 ± 2.0
Lung	Control	3.9 ± 0.33	2.6 ± 0.29
	Smoker	3.6 ± 0.46	2.4 ± 0.35
Spleen	Control	1.5 ± 0.15	2.7 ± 0.35
	Smoker	1.1 ± 0.052	1.9 ± 0.61
Kidney	Control	6.2 ± 0.32	4.5 ± 0.32
	Smoker	6.7 ± 0.80	3.9 ± 0.41
Blood (2)	Control	14.8 ± 0.75	8.8 ± 0.52
	Smoker	13.7 ± 1.6	9.4 ± 1.1

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: + $p<0.05$.

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: * $p<0.05$.

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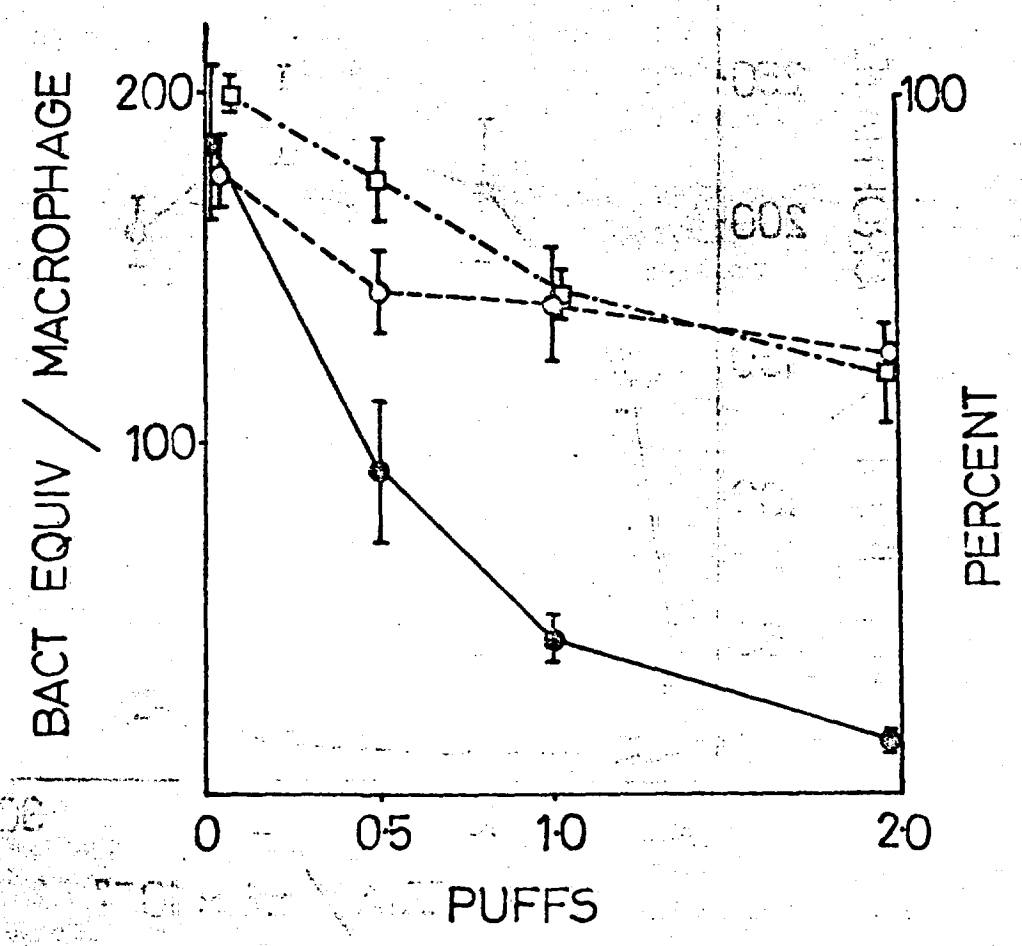

FIGURE 20 Effect of cigarette smoke on viability, adherence and phagocytosis of macrophages *in vitro*. The amount of radioactivity accumulated during 2 hours incubation with radiolabelled *Pseudomonas aeruginosa* was determined as a measure of phagocytic activity. Each result is the mean ± S.E. of the values from 3 cultures.

● phagocytosis, o adherence, □ viability. Viability and adherence (p.34) are shown as percentages of control values and phagocytosis is shown as bact. equivs. phagocytosed.

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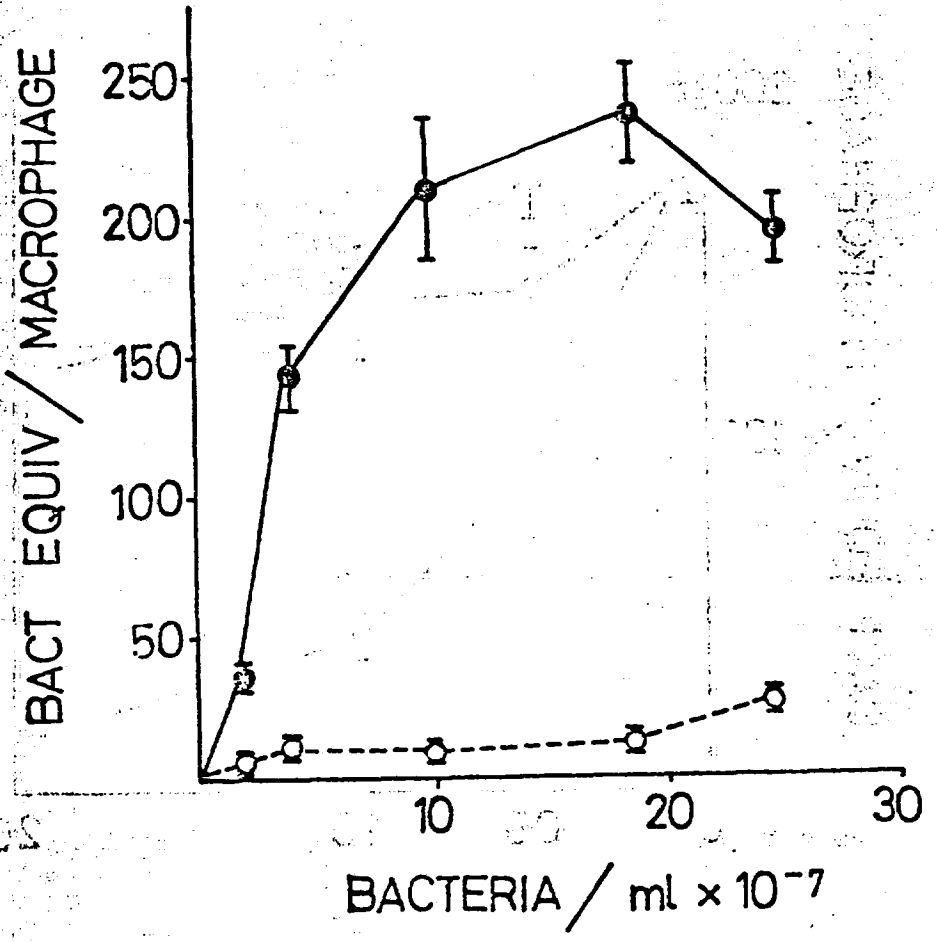

FIGURE 21 Phagocytosis of macrophages exposed to cigarette smoke *in vitro* and incubated for 2 hours in varying concentrations of radiolabelled *Pseudomonas aeruginosa*. ● control, o macrophages exposed to 1 puff of cigarette smoke.

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TABLE 21 The viability and phagocytic activity of macrophages 30 minutes and 24 hours after the exposure to fresh cigarette smoke

	Group	% control(1) 30 minutes	% control(1) 24 hours
Phagocytosis in:	Controls	100.0 ± 2.1	100.0 ± 4.0
	Whole smoke	***21.8 ± 0.96	***28.6 ± 3.5
	Vapour phase	***21.7 ± 3.9	***55.0 ± 5.0
	Whole smoke, washed (2)	***22.9 ± 4.5	***39.7 ± 1.2
Viability	Controls	100.0 ± 3.4	100.0 ± 2.0
	Whole smoke	*56.0 ± 11.0	**50.3 ± 6.7
	Vapour phase	**54.4 ± 11.0	*74.7 ± 7.4
	Whole smoke, washed	*73.3 ± 8.4	***54.0 ± 1.5

(1) The results are expressed as a percentage of the corresponding control value. Each result is the mean ± S.E. of three cultures.

(2) Immediately after smoke exposure the macrophages were washed with three 2 ml changes of PBS.

The significance of the differences between the tests and the controls were determined by Student's t test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

After 24 hours the phagocytosis/macrophage by macrophages exposed to vapour phase or whole smoke and washed was significantly different from cells only exposed to whole smoke ($p<0.01$).

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When the radioactivity accumulated by macrophages phagocytosing for periods up to 5 hours was measured it was found that one puff of the cigarette smoke caused a depression of radioactivity detected after 2 hours but in contrast increased the radioactivity accumulated in 5 hours (Fig. 22). To examine the possibility that the increased accumulation of radioactivity produced by exposure to cigarette smoke could be caused by a defect in the ability of macrophages to degrade and eliminate bacteria, the ability of the macrophages to eliminate phagocytosed bacteria was measured. Macrophages were incubated with bacteria for 3 hours and an enhanced accumulation of radioactivity occurred in the macrophages exposed to cigarette smoke. The elimination of the radioactivity measured over the next three hours was faster than control macrophages (Table 22). The vapour phase of cigarette smoke could also stimulate the accumulation of radioactivity (Table 23). In subsequent experiments it was found that different preparations of the radiolabelled *Pseudomonas aeruginosa* influenced the effect of cigarette smoke. Although it was attempted to standardize the preparations of different batches of bacteria, when some preparations were used the stimulation could be detected after 2 hours of phagocytosis while with others the stimulation could not be detected at 5 hours.

E. Discussion

Previous data has shown that the mice used in these experiments have a larger number of alveolar macrophages (Holt and Keast 1973a) than control mice. The finding that bacteriocidal activity in their lungs was depressed despite the increase in alveolar macrophages concurs with results obtained by Rylander using guinea pigs (Rylander 1972a,b). These results indicate that the bacteriocidal activity of macrophages in the lung was depressed by the inhalation of cigarette smoke. The clearance of the radiolabelled bacteria from the lungs of male mice exposed to cigarette smoke was also found to be depressed.

Previous reports have described the inhibitory effect of cigarette smoke and its vapour phase on the phagocytic activity of macrophages cultivated *in vitro* (Green and Carolin 1967; Green 1968; Lentz and DiLuzio 1972, 1973). The experiments with macrophages

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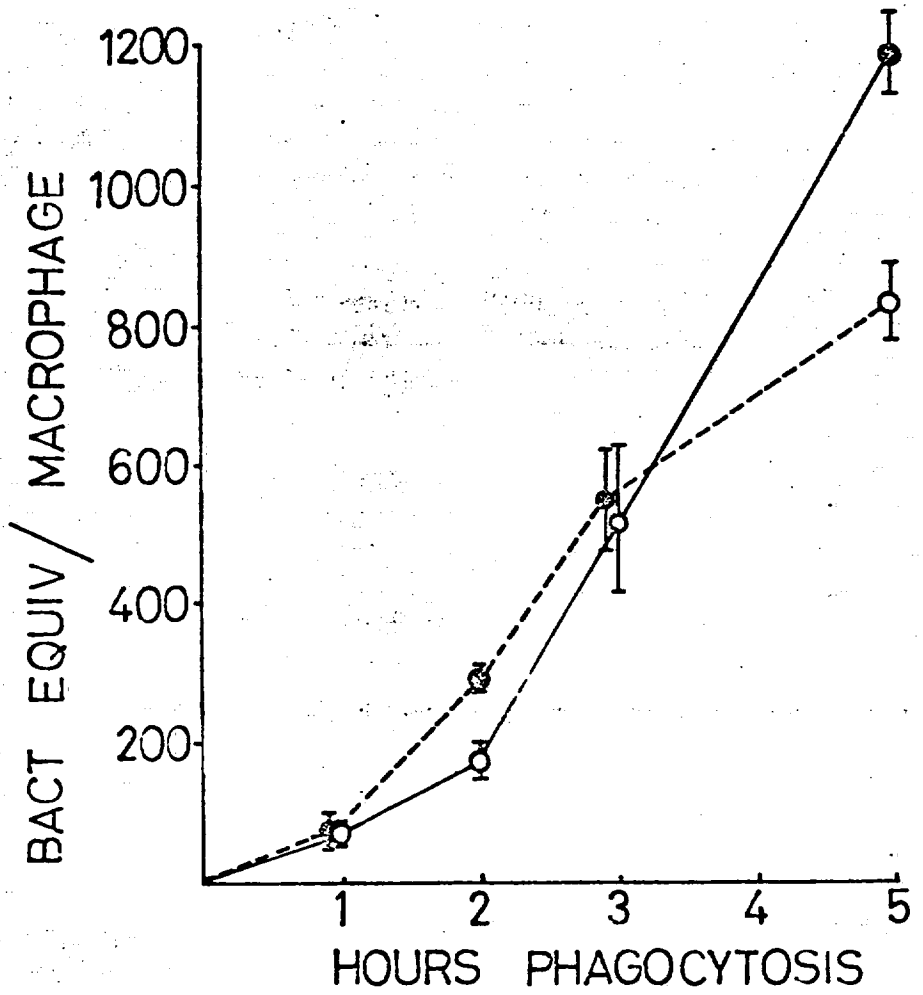

FIGURE 22 Effect of exposure to cigarette smoke on the accumulation of radioactivity from radiolabelled *Pseudomonas aeruginosa* by macrophages *in vitro*. ● control, o macrophages exposed to 1 puff of cigarette smoke. The significance of the difference between control and smoke exposed macrophages was determined by Student's t test. The radioactivity in the test and control macrophages was significantly different at 2 hours ($p<0.02$) and 5 hours ($p<0.01$).

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TABLE 22 The elimination of phagocytosed radiolabelled *Pseudomonas aeruginosa* from cigarette-smoke-exposed macrophages

	Phagocytosis	Elimination	
	(Bact. equiv/cell)	(Bact. equiv/cell)	%(1)
Control	246 ± 40	119	48
Smoke exposed	700 ± 142	280	40

After exposing the macrophages to 1 puff of cigarette smoke the phagocytosis after three hours and the elimination after a further three hours were measured. Triplicate cultures were used (mean ± S.E.)

(1) Percent of the radiolabel phagocytosed.

TABLE 23 The effect of the vapour phase on the accumulation of radiolabelled bacteria by macrophages *in vitro*

Time of phagocytosis	Exposure	
	Control	Vapour phase
2 hours	112 ± 16*	75 ± 3
5 hours	267 ± 23	413 ± 33

* Bacteria equivalents of radiolabel/macrophage.

Each result represents the mean ± S.E. of the values obtained from 3 cultures.

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in vitro described here have used peritoneal macrophages as previous data has shown that cigarette smoke produces similar changes in the RNA and protein synthesis of alveolar and peritoneal macrophages (Holt and Keast 1973b,d). When the macrophages began to phagocytose bacteria after exposure to cigarette smoke their rate of accumulation of radioactivity was depressed, but they were eventually able to accumulate radioactivity more rapidly than control macrophages. This phenomena did not appear to be caused by a defect in the ability of the macrophages to digest and eliminate the radioactivity. Metabolically inactive macrophages are more susceptible to toxic effects of cigarette smoke (Holt and Keast 1973b). It is possible that macrophages with a low phagocytic rate could be killed by the cigarette smoke leaving a population with a higher average phagocytic rate. This was not the only factor as the total phagocytosis of a monolayer of macrophages exposed to cigarette smoke often exceeded the total phagocytosis of a control monolayer. The vapour phase could also produce both the stimulation and inhibition of phagocytosis. It is not known how the different preparations of *Pseudomonas aeruginosa* slightly influenced the results as it was attempted to standardize the preparation of bacteria.

The results in these experiments showed that the macrophages accumulated large amounts of bacteria. This was consistent with morphological observations and the fact that continued phagocytosis was toxic for the macrophages (Thomas, Holt and Keast 1974b). Without allowing for digestion of the phagocytosed bacteria, the volume of bacteria accumulated by the macrophages reached about 20% of the volume of the macrophages. The stimulatory effect of endotoxins from the bacteria (Stuart 1970) may account for large amounts of phagocytosis. It is also possible that the digestion of bacteria within macrophages decreased the self-absorption of radioactivity by the bacteria and this exaggerated the measurement of phagocytosis.

Macrophages incubated without bacteria recovered slowly from the inhibitory effect of cigarette smoke on their phagocytic activity. The difference in the rate of recovery of the macrophages exposed to whole smoke and vapour phase indicated that more than one component in cigarette smoke inhibits phagocytosis.

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In experiments reported by Green (1968), macrophages cultured in medium containing fresh serum, inactivated 70% of bacteria added to the cultures, while macrophages incubated in medium without serum only inactivated 43% of the bacteria. However macrophages which had been exposed to cigarette smoke could inactivate 49% of the bacteria in the absence of serum, but only 1% of the bacteria in the presence of serum. The absence of serum may correspond to the heat-treated serum used for the experiments described in this section. It is apparent that the direct effect of cigarette smoke on macrophages is complex, but when evaluating the effect of cigarette smoke on phagocytosis it is important to consider that relatively small doses of cigarette smoke were highly toxic for macrophages and caused considerable cell death.

The clearance of radiolabelled bacteria from the blood, as measured by the phagocytic index, was unaffected in mice chronically exposed to cigarette smoke. However 10 minutes after the inoculation of bacteria the livers of mice chronically exposed to cigarette smoke contained considerably less radioactivity than the livers of control mice. The decrease of radioactivity in the liver from 10 to 180 minutes was greatest in the control animals. Other interpretations of these results are possible, but the results strongly suggest that the phagocytic and degradative activity of the liver phagocytes was impaired by the inhalation of cigarette smoke. The most obvious source of radioactivity in the kidney was material phagocytosed, degraded and eliminated from phagocytic cells. This radioactivity was increased in mice exposed to the cigarette smoke, even when the radioactivity in the liver was the same as the control. This would mean that another phagocytic component with a rapid rate of digestion of the bacteria was operating in mice exposed to cigarette smoke. One possibility is the circulating polymorphonuclear leucocytes. A decrease in liver function may mean that its important detoxifying action on endotoxins in the portal blood is decreased (Thomas, McSween and White 1973). This could have important effects on the immune system and the health of the animal, as will be discussed in Chapter 5.

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SECTION 4 GROWTH OF TRANSPLANTED TUMOURS

A. Introduction

It was considered that if immunosuppression, produced by the inhalation of cigarette smoke, could allow neoplasms to escape from immunological control (Holt, Thomas and Keast 1973; Keast and Holt 1974), it should be possible to demonstrate that growth of established immunogenic neoplasms in mice is enhanced by cigarette-smoke inhalation. Two experimental systems were used to examine this possibility: (i) the ability of the Lewis lung tumour to grow subcutaneously and mestastasise to lungs, (ii) the growth of intra-tracheally-inoculated TKL5 tumour cells.

The Lewis lung tumour originated as a spontaneous lung carcinoma in a C57Black mouse and was routinely maintained by serial passage in adult mice. Immunostimulants have been shown to inhibit the growth of this tumour (Renoux and Renoux 1972). The TKL5 tumour cells were from a tumour induced by a murine sarcoma virus (see Chapter 2 section M) and were maintained in tissue culture. These cells did not readily grow in adult mice. The antigenicity of tumours induced by murine sarcoma viruses is well documented (Fefer, McCoy and Glynn 1967; Law, Ting and Stanton 1968).

B. Subcutaneous growth and metastasis of Lewis lung tumour

C57Black mice were exposed to fresh cigarette smoke for 3 days, 23 weeks and 38 weeks prior to the subcutaneous inoculation of 10^6 Lewis lung tumour cells. Cigarette-smoke exposure was continued and the tumour growth was estimated by measuring diameters. The growth of tumours in mice exposed to cigarette smoke for three days before the inoculation was almost identical to the growth of tumours in control mice (Fig. 23). However the growth of the tumours in mice that had been exposed to cigarette smoke for 23 and 38 weeks was significantly increased as compared to control animals (Fig. 23). The lungs were examined for metastases. The mice exposed to cigarette smoke for 23 and 38 weeks had 2.8 ± 1.1 (Mean ± S.E.) while age-matched control mice had 0.8 ± 0.4 metastases per lung.

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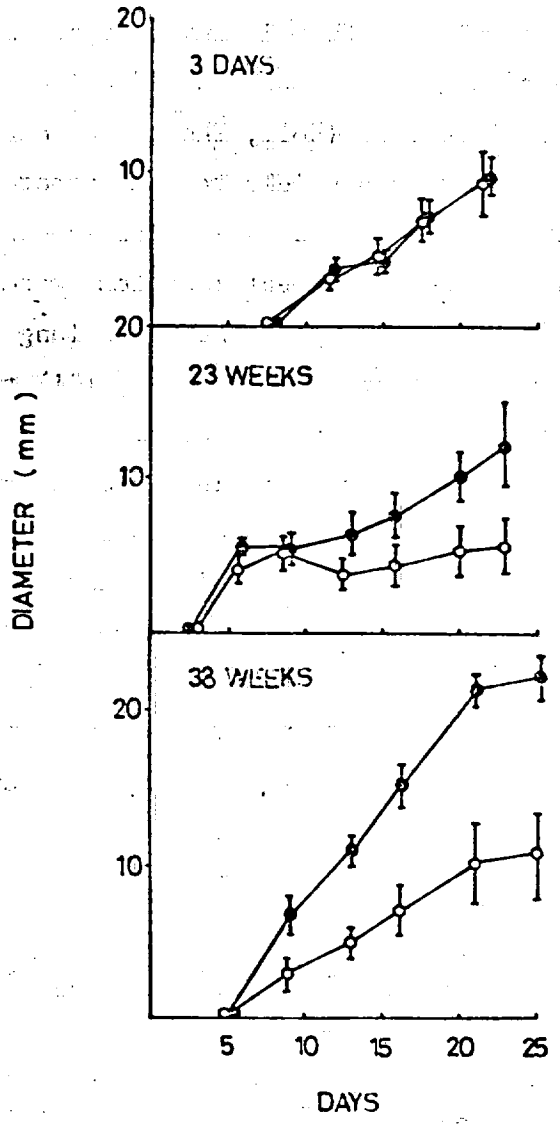

FIGURE 23 The subcutaneous growth of the Lewis lung tumour in mice exposed to cigarette smoke for 3 days, 23 weeks and 38 weeks. Each result is the mean ± S.E. of the tumour diameter of 10 mice. Smoke exposed ●, Control o. As determined by Student's t test there was no significant difference between the tumour growth in mice exposed to cigarette smoke for 3 days and controls but the diameter of the tumours in mice exposed to cigarette smoke for 23 and 38 weeks became significantly different from their corresponding controls 20 and 9 days after the tumour inoculation, respectively.

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C. Growth of intratracheally-inoculated tumour cells

BALB/c mice were exposed to cigarette smoke for 20 weeks and then inoculated intratracheally with 10^5 TKL5 tumour cells. As shown in Table 24 (Exp. 1), 6/13 of the smoke-exposed mice died or became moribund. None of the control mice died within 50 days. In another experiment (Table 24 Exp. 2) a group of BALB/c mice were exposed to cigarette smoke for 31 weeks before receiving the intratracheal inoculation of TKL5 cells. As well as a group of control mice, which were not exposed to cigarette smoke, this experiment also included a group of mice, the same age, but only exposed to cigarette smoke on the 3 days preceding the inoculation. Only mice exposed to cigarette smoke for 31 weeks died or became moribund (6/15). The lungs of mice which died after the inoculation of tumour cells were examined histologically. They exhibited numerous foci of neoplastic proliferations varying in size from a few cells to large extensive zones of neoplastic growth (Fig. 24) which completely replaced the whole lobes of the lungs. Generally the foci were small, consisting of 50 - 200 cells and were situated near bronchi or small vessels. Mitoses were present. In the cases where foci were large, vasoproliferation became evident together with an infiltrate of lymphocytes, macrophages and giant cells.

D. Discussion

The results show that the subcutaneous growth of the transplantable Lewis lung tumour was significantly increased in mice chronically exposed to cigarette smoke. Mice chronically exposed to cigarette smoke showed a higher number of metastases in the lung than control mice, but this difference was not statistically significant. However the TKL5 tumour cells only grew significantly in the lungs of mice chronically exposed to cigarette smoke. The experiments measuring the clearance of intratracheally-inoculated bacteria (Chapter 4 section 3.B) indicate that depression in tracheobronchial-clearance activity was not responsible for increasing the susceptibility of mice exposed to cigarette smoke to intratracheally - inoculated tumour cells.

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TABLE 24 The response of cigarette-smoke-exposed mice to an intratracheal inoculation of TKL5 tumour cells

	Smoke Exposure	No. dead* / No. inoculated	Days to death Mean (range)
Exp. 1	20 weeks	6/13	28.6 (14-40)
	Control	0/19	
Exp. 2	31 weeks	6/15	15.0 (10-46)
	3 days	0/20	
	Control	0/20	

* Number of mice dead or moribund.

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The experiments indicate that inhalation of cigarette smoke was able to modify conditions within the smoking animal that result in the promotion of growth of an established malignant cell. The fact that conditions for the production of enhanced tumour growth did not occur after short term exposure to cigarette smoke (3 days) indicated that either an accumulation of substances acting directly on tumour cells must occur or that cigarette-smoke inhalation eventually impairs a mechanism controlling tumour growth. These results are consistant with the concept that immunosuppression, induced by the inhalation of cigarette smoke, allows immunogenic neoplastic cells to escape from immune control.

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CHAPTER 5

GENERAL DISCUSSION

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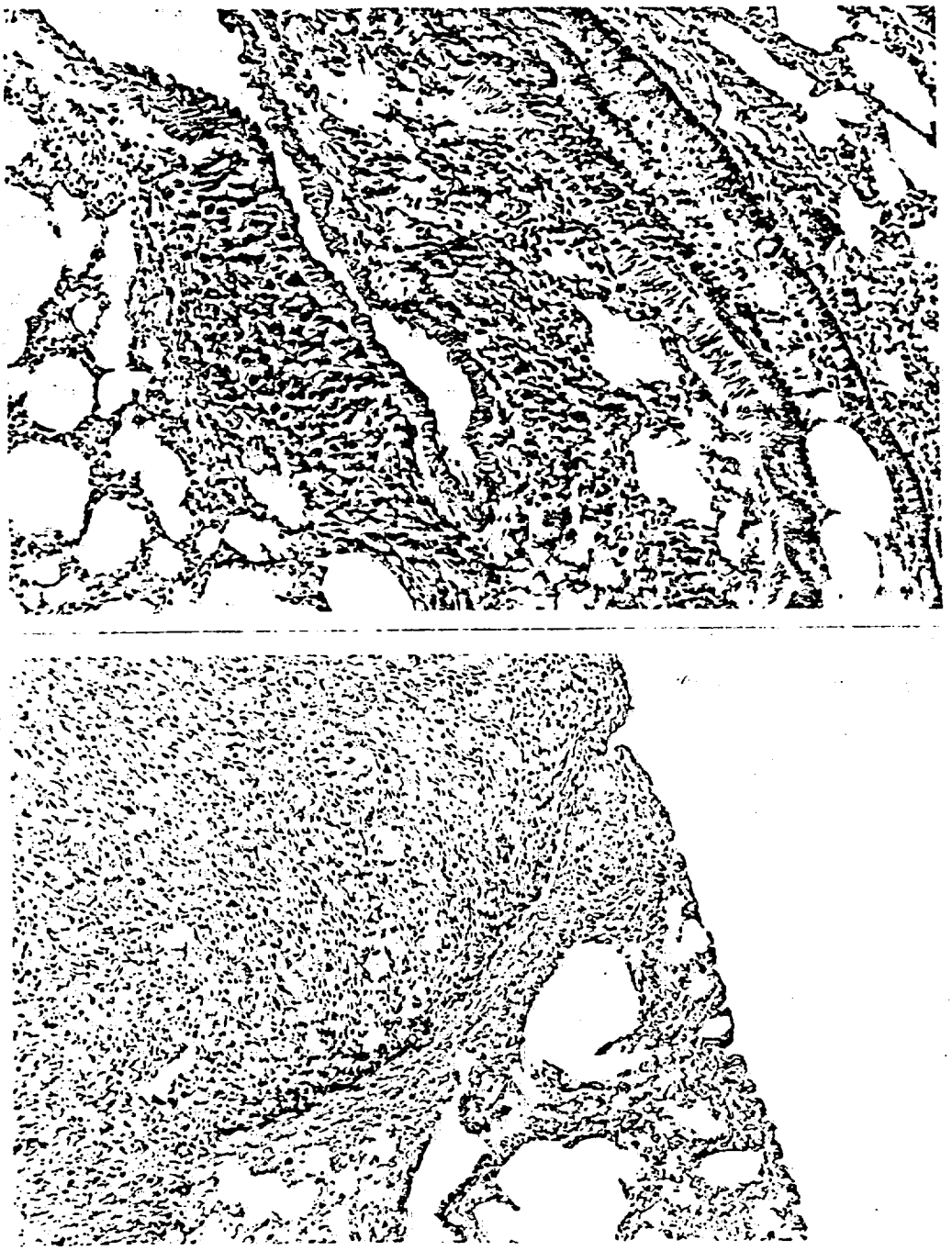

FIGURE 24 Growth of intratracheally-inoculated TKL5 cells in the lungs of mice exposed to cigarette smoke.

TOP: Aggregates of large malignant cells are found in the near vicinity of an arteriole and a small bronchiole. A mononuclear infiltrate surrounds the aggregates. Magnification 140 X.

BOTTOM: A large neoplastic mass has completely replaced much of the parenchyma of the lung. Magnification 87 X.

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The Second World Conference on Smoking and Health concluded that there was a need to develop experimental models to assist in producing less harmful cigarettes and determining how cigarette smoking produces disease (Workshop of the Second World Conference on Smoking and Health 1972). An understanding of the mechanism(s) by which cigarette smoking produces disease could be useful in identifying cigarette smokers with a high risk of developing disease, in the treatment and prophylaxis of the diseases associated with cigarette smoking and for developing bioassay systems for evaluating the safety of tobacco products and substitutes. The mechanism(s) of action of cigarette smoke on experimental models should relate to the mechanism(s) of producing disease in humans.

It has also become apparent, because of the aging of cigarette-smoke components (Hoffmann and Wynder 1970), the effects of components of the vapour phase of cigarette smoke (Leuchtenberger and Leuchtenberger 1970a) and the method used by humans to administer cigarette smoke to themselves, that the experimental models should involve the inhalation of fresh cigarette smoke (Dontenwill 1970; Hoffmann and Wynder 1970; Leuchtenberger and Leuchtenberger 1970). Active inhalation experiments have been performed where animals inhale air through a burning cigarette (Albert *et al* 1969; Auerbach *et al* 1970a), but these techniques require large animals and difficulties are encountered when experiments require the use of large numbers of animals (Hoffmann and Wynder 1970). Hence inhalation chambers, like the Hamburg II smoking apparatus, have been developed in order to expose laboratory rodents to cigarette smoke. The development of animal models for studying biological effects of cigarette smoking, as well as attempting to simulate the smoking habit, also represent attempts to reproduce disease processes which may take 20 - 30 years to develop in human smokers (Royal College of Physicians 1971; United States Public Health Service 1971).

An examination of the immune system of animals exposed to cigarette smoke, as well as the immune system of human smokers may be important in determining both the suitability of the animal as a model for studying diseases associated with cigarette smoking and the role of immune dysfunction, if any, in the aetiology of these diseases. The influence of cigarette smoking on immunity is

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pertinent because immunosuppression may, at least in part, contribute to the aetiology of the elevated incidence of infection and neoplasia associated with cigarette smoking (Chapter 1 section A). While the relevance of immunological changes, produced in animals by the inhalation of cigarette smoke, to human disease can only be determined after investigations into the immune system of human smokers, results of studies with inbred animals can be used as a guide for designing studies of human smokers.

In the experiments described in Chapters 2, 3 and 4, inbred mice were exposed to cigarette smoke in a Hamburg II smoking apparatus (Dontenwill 1970) for 7 minutes on five days of each week for periods up to 42 weeks. Although the inhalation of cigarette smoke produced some depression in the appetite, body weight and haemoglobin concentration in the mice the changes were not large and the mice remained apparently healthy during the experiments (Chapter 2).

The primary humoral immune responses of mice to SRBC, an immunogen requiring T-cells, (Davies *et al* 1971; Playfair 1971), were transiently enhanced by the inhalation of cigarette smoke but after chronic inhalation of smoke these immune responses were markedly depressed (Chapter 4 sections 1.C and 1.D; Thomas, Holt and Keast 1973a, 1974c). The depressions were evident when SRBC were inoculated intraperitoneally or intratracheally, and were shown by decreases in the antibody-forming cell response in the cervical and mediastinal lymph nodes, lungs and spleen as well as serum antibody titres. These changes were at least partially reversible by discontinuing smoking (Chapter 4 section 1.C; Thomas, Holt and Keast 1974d). However serum antibody responses to PVP, an immunogen not requiring T-cells (Andersson and Blomgren 1971), were not enhanced or depressed by inhalation of cigarette smoke (Chapter 4 section 1.F). This indicates that cigarette smoke had little direct effect on the antibody-producing cells and antibody in mice, a supposition supported by experiments with antibody and antibody-forming cells *in vitro* (Chapter 4 section 1.G), and the finding that short term inhalation of cigarette smoke did not decrease the humoral immune responses to SRBC (Chapter 4 section 1.C and 1.D; Thomas, Holt and Keast 1974c).

The transient stimulation of the humoral immune response to SRBC by cigarette smoke did not appear to be mediated by infection or exposures to an agent antigenically related to SRBC as the number

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of antibody-forming cells in the spleen and lymph nodes of mice which were unimmunized but exposed to cigarette smoke was the same as in unimmunized control mice (Chapter 4 section 1.E). Esber *et al* (1973) did not show a transient enhancement in the humoral immune response of mice, exposed to cigarette smoke, to SRBC. Instead, after only five days exposure to the cigarette smoke, mice had severely depressed primary and secondary haemagglutinating antibody responses to intraperitoneal inoculations of SRBC. However the regime of exposure to cigarette smoke used by Esber *et al* (1973) was considerably more severe than the exposures used in the present experiments (Chapter 3).

The ability of mice, chronically exposed to cigarette smoke to effect cell-mediated immune reactions, as judged by the responsiveness of their lymphocytes to PHA, was impaired (Chapter 4 section 2.B; Thomas, Holt and Keast 1973b). However the responsiveness of lymphocytes from cervical and mediastinal lymph nodes to PHA was transiently enhanced by the inhalation of cigarette smoke, similar to the immune responses to SRBC. These reactions have also been shown to be accompanied by a transient infiltration of lymphocytes into the lungs of mice (Holt and Keast 1973a). The similarities in the changes occurring in the responsiveness of lymphocytes from mice exposed to cigarette smoke to PHA and antibody responses to an immunogen requiring T-cells, SRBC, suggest that inhalation of cigarette smoke produces a defect in T-cell function. Experiments showing that antibody responses, to PVP, an immunogen not requiring T-cells, were not influenced by inhalation of cigarette smoke, support this concept.

When macrophages were exposed to cigarette smoke *in vitro* and then incubated with bacteria their initial phagocytic activity was depressed, but if the incubation with bacteria was continued the phagocytic activity of the macrophages was higher than the phagocytic activity of control macrophages (Chapter 4 section 3.D). The ultimate effect of inhalation of cigarette smoke on the function of alveolar macrophages was estimated by determining the bacteriocidal activity in the lungs of mice (Chapter 4 section 3.B). Pulmonary bacteriocidal activity, which has been attributed to the activity of alveolar macrophages (Green and Kass 1964a,b), was depressed in mice chronically exposed to cigarette smoke. This depression occurred

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despite the fact that mice exposed to cigarette smoke had increased numbers of alveolar macrophages (Holt and Keast 1973a). Experiments conducted by Rylander (1971a,b) similarly showed that guinea pigs, exposed to cigarette smoke, had depressed pulmonary bacteriocidal activities in the presence of elevated numbers of alveolar macrophages. Human cigarette smokers have also been shown to have a higher number of alveolar macrophages than nonsmokers. However when alveolar macrophages were cultivated *in vitro*, their phagocytic activity was similar to that of alveolar macrophages cultivated from nonsmokers (Cohen and Cline 1971; Harris *et al* 1971; Mann *et al* 1971). It is possible that the measurement of phagocytic capacity of alveolar macrophages *in vitro* may not be a sufficient measure of bacteriocidal activity in the lungs. Alterations in phagocytic cells could also account for other changes in the immune response produced by cigarette smoke inhalation. The influence of macrophages on the antibody response to SRBC is well documented (Ford, Gowans and McCullough 1967; Mosier 1967; Shortman *et al* 1970; Cruchard and Unanue 1971).

The function of liver phagocytes was also apparently depressed in the mice chronically exposed to cigarette smoke (Chapter 4 section 3.L). This impairment could decrease the removal of endotoxins from portal blood (Thomas, McSween and White 1973; Woodruff, O'Carroll, Koizumi and Fine 1973), which have been shown to be capable of either enhancing or inhibiting the immune response (Franzl and McMaster 1968; Neter 1969; Chedid 1973; Jutila 1973). Laurenzi, Gaurneri and Endriga (1965) have shown that either enteric bacteria or an intraperitoneal inoculation of endotoxin was required to allow acute exposures of cigarette smoke to inhibit the removal of viable bacteria from mouse lungs. There is also evidence suggesting that endotoxins can weaken the immunosurveillance mechanisms against neoplasms (Stanley and Keast 1967; Keast 1968; Keast, Stanley and Phillips 1968; Keast and Stanley 1969). However no leucocytosis was found in the mice as might be expected from the actions of endotoxin (Jutila 1973). Substances found in cigarette smoke, carbon particles and cadmium (Lewis, Jusko, Coughlin and Hartz 1972), have also been shown to be capable of both enhancing or inhibiting immune responses (Jones, Williams and Jones 1971; Zarkower 1972a,b).

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The exposure to cigarette smoke involves forcing the mice to inhale an irritant. While the physical handling of the mice has been found not to influence humoral immune responses (Esber *et al* 1973) or body weights (Leuchtenberger and Leuchtenberger 1970a) it is difficult to evaluate the effect of the stress produced by compelling the mice to inhale the smoke. The problem has been approached by examining temporal aspects of the alterations produced in the immune system. In particular it was thought that changes occurring soon after commencing cigarette smoke exposure were likely to be due to stress; during this time the mice displayed movements indicative of discomfort. Except for the pfc response in the lungs of mice after intratracheal inoculations of SRBC, no depressions in the immune response were found before 20 weeks of exposure to cigarette smoke, however some immune responses were enhanced (Chapter 4 section 1.C and 1.D). It appears improbable that the depressions in immune responses ensuing in the following 20 weeks should be due to stress, especially when the mice had become accustomed to smoke exposure. A similar sequence of transient enhancement and then depression in immune responses has been noted in mice exposed to carbon and oxidant gas (Zarkower 1972a,b).

Although primary immune responses of mice exposed to cigarette smoke to SRBC were depressed, it was apparent that mice could mount large immune responses to continued antigenic challenge. This poses the question whether or not the immunodeficiencies noted in mice exposed to cigarette smoke can influence the resistance of mice to neoplasia and infection. Similarly the phagocytic activity in the lungs of mice exposed to cigarette smoke, although less efficient than control mice, was effective in inactivating large numbers of bacteria (Chapter 4 section 3.B). The results of experiments described in this thesis (Chapter 4 section 4.B and 4.C) showed that the growth of established malignant cells was increased in mice exposed to cigarette smoke, when compared to their growth in age-matched-control mice. Spurgash *et al* (1968) have shown that mice exposed to cigarette smoke are also more susceptible to respiratory infection. Studies on the influence of inhalation of cigarette smoke on pulmonary tumorigenesis in mice exhibiting a relatively high natural incidence of lung adenomas and adenocarcinomas, showed an earlier onset and increased incidence of these neoplasms (Leuchtenberger and Leuchtenberger 1970a; Leuchtenberger, Leuchtenberger and Rossier

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1973). However, when a strain of mice with a lower natural incidence of these neoplasms was examined, under the same experimental conditions, no increase in the incidence of neoplasms was found (Leuchtenberger, Leuchtenberger and Rossier 1973). These studies are consistant with proposals that inhalation of cigarette smoke produces a significant depression in the ability of the immune system of mice to control neoplastic growth and infection.

There are also indications that cigarette smoking impairs the ability of the human immune system to control infection and neoplasia. Cigarette smokers have been found to have a less persistant serum antibody response to influenza virus infection and vaccination than nonsmokers (Finklea, Hasselblad, Riggan, Nelson, Hammer and Newill 1971). Alveolar macrophages from cigarette smokers, but not from nonsmokers, have been reported to be refractile to MIF (Macrophage Migration Inhibition Factor), a product produced by activated lymphocytes, which probably has an important function in recruiting macrophages into cell-mediated immune reactions (Warr and Martin 1973b). Cigarette smokers have a higher number of alveolar macrophages than nonsmokers (Harris *et al* 1970a), similar to the increased number of alveolar macrophages found in mice and guinea pigs exposed to cigarette smoke (Rylander 1971a; Holt and Keast 1973a), but when alveolar macrophages from smokers were cultivated *in vitro* their phagocytic activity was similar to that of macrophages from nonsmokers (Harris *et al* 1971a; Mann *et al* 1971; Cohen and Cline 1971). As both mice and guinea pigs exposed to cigarette smoke have decreased pulmonary bacteriocidal activities, despite elevated numbers of alveolar macrophages, it is possible that cigarette smokers, who also have elevated numbers of alveolar macrophages, have depressed pulmonary bacteriocidal activity and that measurement of phagocytic activity *in vitro* does not reflect phagocytic activity *in vivo*. A discrepancy exists between the effect of cigarette smoke on the peripheral blood leucocytes of mice and humans. Cigarette smokers have a higher number of peripheral blood leucocytes than nonsmokers (Howell 1970; Corre *et al* 1971; Friedman *et al* 1973) but exposure to cigarette smoke decreases the number of peripheral blood leucocytes in mice (Chapter 3 section F). Until the role of infection in elevating leucocyte counts of human smokers is determined (Friedman *et al* 1973) these differences cannot be fully evaluated.

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Although the immunosuppressive effect of cigarette smoke may easily be considered to account for the increased respiratory infection of cigarette smokers, the participation of immunosuppression, if any, in the aetiology of the human lung cancer attributed to cigarette smoking may be complex. Mice chronically exposed to cigarette smoke have an increased incidence of adenocarcinoma (Harris and Negroni 1967; Leuchtenberger and Leuchtenberger 1970a), which is the common pulmonary neoplasm of mice (Stewart, Dunn and Snell 1970). Adenocarcinomas are also the most frequent human lung cancer (Berg 1970), but these neoplasms constitute only a small proportion of lung cancer attributed to the effects of cigarette smoking (United States Public Health Service 1971; Royal College of Physicians 1971). Furthermore, filters have been shown to increase the carcinogenic activity of cigarette smoke in mice (Leuchtenberger and Leuchtenberger 1970) but decrease the incidence of lung cancer in humans (Royal College of Physicians 1970; United States Public Health Service 1971). These findings support proposals implicating an immunosuppressive effect, of a substance in the vapour phase of cigarette smoke, in producing the increased incidence of lung cancer in mice and the action of carcinogens in the particulate phase of cigarette smoke in producing human lung cancer. However the role of carcinogens in cigarette smoke in initiating human lung cancer is not clear. The high incidence of lung cancer in uranium and asbestos workers appears to be dependent on cigarette smoking but the occupational contaminants appear, at least in part, to determine the histology of the cancers (Selikoff, Hammond and Churg 1968; Churg and Kannerstein 1970; Archer *et al* 1973). There is also some evidence indicating that common atmospheric pollutants may initiate a proportion of lung cancers which are then promoted by cigarette smoking (Cannow and Meier 1973). While it is well documented that cigarette smoke contains substances which can initiate and promote carcinogenesis (Wynder and Hoffmann 1964; Stedman 1967; United States Public Health Service 1971), there may be many instances when carcinogenesis is initiated by an agent other than cigarette smoke but the tumour promoting activity of cigarette smoke is required for the development of lung cancer. Immunosuppression, induced by cigarette smoking, may be responsible for promoting carcinogenesis

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induced by carcinogens from cigarette smoke or other sources.

There are also several possible inter-relationships between infection, immunosuppression and carcinogenesis which may be relevant to the diseases found in cigarette smokers. Respiratory tract infection can decrease the bacteriocidal activity in the lungs (Green 1965; Klein *et al* 1968; Jakab and Green 1973). This would provide a mechanism to amplify the immunosuppression induced by exposure to cigarette smoke. Viruses could exert a cocarcinogenic effect by changing the metabolism of cells to render them more susceptible to carcinogens or by producing immunosuppression; the infection of animals with virus has been shown to increase their susceptibility to chemical carcinogens (Kotin and Wisely 1963; Southam *et al* 1969; Nettesheim, Richters and Liziby 1972). However virus infection has also been shown to decrease the adenomas and adenocarcinomas produced in X-radiated mice with chemical carcinogens (Nettesheim *et al* 1970). The incidence of atypical bronchial proliferations produced by cigarette smoke has also been found to be increased by influenza infection (Leuchtenberger *et al* 1963). The role of the metaplastic changes in the production of cancer is not known (Saccomanno *et al* 1970) but chronic infection with parainfluenza virus or mycoplasma can produce metaplastic lesions resembling squamous cell carcinomas (Richter 1970).

A depressed immune system could also facilitate the activity of oncogenic viruses. The interaction of oncogenic viruses and cigarette smoke has been considered (Leuchtenberger, Leuchtenberger, Zebrun and Schaffer 1960; Leuchtenberger 1970). Condensate from cigarette smoke has been shown to induce C-type oncogenic viruses from cells *in vitro* (Freeman *et al* 1971) and infection with C-type virus has been shown to render cells more susceptible to transformation with chemical carcinogens (Rhim *et al* 1972; Saleno, Ramm and Whitmire 1973).

As outlined above, several factors may be involved in the aetiology of lung cancer in cigarette smokers. Immunosuppression may contribute to both the induction and promotion of lung cancer in cigarette smokers and animals exposed to cigarette smoke, as well as respiratory infection. Experiments with animals and investigations of cigarette smokers have shown that cigarette smoke can damage immune function.

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